

FORM-PTO-1390 (Rev. 5-93)		U. S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 2325-1-002
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U. S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/180657
INTERNATIONAL APPLICATION NO. PCT/AU97/00304	INTERNATIONAL FILING DATE 16 May 1997	PRIORITY DATE CLAIMED 17 May 1996	
TITLE OF INVENTION EARLY DETECTION OF LYSOSOMAL STORAGE DISORDERS			
APPLICANT(S) FOR DO/EO/US Peter J. MEIKLE; Douglas A. BROOKS; John J. HOPWOOD			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).</p> <p>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p>a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</p> <p>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)</p> <p>6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p>a. <input checked="" type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input checked="" type="checkbox"/> have been transmitted by the International Bureau.</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p>d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An executed oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>Items 11. to 16. below concern other document(s) or information included:</p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p><input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification.</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>16. <input checked="" type="checkbox"/> Other items or information:</p> <p>2 SMALL ENTITY STATEMENTS; TWELVE (12) SHEETS OF FIGURES; COPY OF INTERNATIONAL SEARCH REPORT; COPY OF PRELIMINARY EXAMINATION REPORT; COPY OF WRITTEN OPINION</p>			

EXPRESS MAIL "MAILING CERTIFICATE NO." : EL149348085US DATE OF DEPOSIT: NOVEMBER 12, 1998

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.50)		INTERNATIONAL APPLICATION NO PCT/AU97/00304		ATTORNEY'S DOCKET NUMBER 2325-1-002	
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17. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO \$930.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) \$720.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) \$790.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$1,070.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) \$ 98.00 <div style="text-align: right;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div>					
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	75 -20 =	55	X \$22.00	\$ 1,210.00	
Independent Claims	2 -3 =	0	X \$82.00	\$.00	
Multiple dependent claim(s) (if applicable)			+ \$270.00	\$ 270.00	
TOTAL OF ABOVE CALCULATIONS =				\$ 2,550.00	
Reduction for 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$ 1,275.00	
SUBTOTAL =				\$ 1,275.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
TOTAL NATIONAL FEE =				\$ 1,275.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$ 40.00	
TOTAL FEES ENCLOSED =				\$ 1,315.00	
				Amount to be:	
				refunded	\$
				charged	\$

a. ☒ A check in the amount of \$ 1,315.00 to cover the above fees is enclosed.

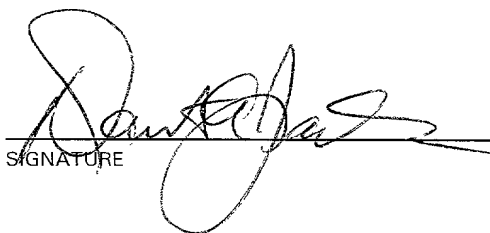
b. ☐ Please charge my Deposit Account No. 11-1153 in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 11-1153. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

DAVID A. JACKSON
KLAUBER & JACKSON
411 HACKENSACK AVENUE
4TH FLOOR
HACKENSACK, NEW JERSEY 07601


 SIGNATURE

 NAME
David A. Jackson
 REGISTRATION NUMBER 26,742

EXPRESS MAIL "MAILING CERTIFICATE NO." : EL149348085US DATE OF DEPOSIT: NOVEMBER 12, 1998

09/11/1998
305 Rec'd PCT/PTO 12 NOV 1998

PATENT
2325-1-002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS : Peter J. MEIKLE; Douglas A. BROOKS; John J. HOPWOOD
APPLICATION NO. : PCT/AU97/00304
FILED : 16 May 1997
FOR : EARLY DETECTION OF LYSOSOMAL STORAGE
DISORDERS

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
BOX PCT
WASHINGTON, D.C. 20231

Sir:

Prior to calculating the fees pursuant to the entry into the National Phase of the above-identified Application, please amend the claims as follows:

IN THE CLAIMS:

In Claim 9, line 1, delete "or 8".

In Claim 12, line 1, delete "any one of claims 1 to 11" and insert --Claim 1--.

In Claim 13, line 1, delete "any one of claims 1 to 11" and insert --Claim 1--.

In Claim 16, line 1, delete "any one of claims 1 to 15" and insert --Claim 1--.

In Claim 19, line 1, delete "any one of claims 1 to 18" and insert --Claim 1--.

In Claim 22, lines 1 and 2, delete "any one of claims 1 to 21" and insert --Claim 1--.

In Claim 37, line 1, delete "any one of claims 30 to 36" and insert --Claim 30--.

In Claim 42, line 1, delete "any one of claims 30 to 41" and insert --Claim 30--.

In Claim 46, line 1, delete "any one of claims 30 to 45" and insert --Claim 30--.

In Claim 48, line 3, delete "any one of claims 1 to 21" and insert --Claim 1--.

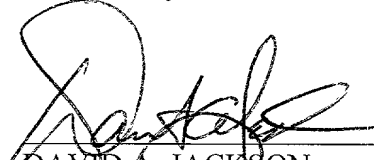
REMARKS

The above amendments are submitted herewith to reduce multiple dependencies and to conform the claims more closely to U.S. practice.

The amendments made herein are with respect to amended Claims 1-51 included in the Written Opinion submitted during the pendency of the International Application. A copy of the Written Opinion dated February 9, 1998, which included the amended claims, is enclosed herewith for your reference.

Entry of the foregoing amendments and early and favorable processing in the National Phase before the United States Patent and Trademark Office is courteously solicited.

Respectfully submitted,



DAVID A. JACKSON
Attorney for Applicant(s)
Registration No. 26,742

KLAUBER & JACKSON
411 Hackensack Avenue
Hackensack, NJ 07601
(201) 487-5800



63 Rec'd PCT/PTO 03 DEC 1998

PATENT

Attorney Docket No.2325-1-002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT : Peter J. Meikle; Douglas A. Brooks; John J. Hopwood
SERIAL NO. : 09/180,657 EXAMINER : Not Assigned
FILED : November 12, 1998 ART UNIT : Not Assigned
FOR : EARLY DETECTION OF LYSOSOMAL STORAGE DISORDERS

CERTIFICATE OF MAILING UNDER 37 CFR 1.8

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to the ASSISTANT COMMISSIONER OF PATENTS, WASHINGTON, DC 20231 on November 30, 1998.

David A. Jackson, Reg. No.26,742
(Name of Registered Representative)

David A. Jackson 11/30/98
(Signature and Date)

SUPPLEMENTAL AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

Dear Sir:

Supplementing our Preliminary Amendment filed concurrently with the submission of the above-identified Application, please amend as follows:

IN THE CLAIMS:

Please delete the amendment to Claim 22 presented in said Preliminary Amendment.

Please amend Claim 22 as follows:

In Claim 22, lines 2 and 3, delete "any one of claims 1 to 21" and insert

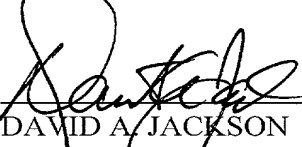
--Claim 1--.

REMARKS

The above Amendment is submitted herewith to correct an inadvertent error that was made in the first filed Preliminary Amendment with respect to Claim 22. The Amendment submitted herewith is believed to be acceptable as no issues of new matter or other material alterations to the application are sought hereby.

Favorable consideration and any action on the merits are courteously solicited.

Respectfully submitted,



DAVID A. JACKSON
Attorney for Applicant
Registration No. 26,742

KLAUBER & JACKSON
411 Hackensack Avenue
August 4, 1998 Hackensack, NJ 07601
(201) 487-5800

Applicant or Patentee: WOMEN'S AND CHILDREN'S HOSPITAL
Serial or Patent No.: _____
Filed or Issued: _____
Title: EARLY DETECTION OF LYSOSOMAL STORAGE DISORDERS

Attorney's
Docket No.: 2325-1-002

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(d) & 1.27(c))--SMALL BUSINESS CONCERN

I hereby declare that I am

☐ the owner of the small business concern identified below:

☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF SMALL BUSINESS CONCERN WOMEN'S AND CHILDREN'S HOSPITAL

ADDRESS OF SMALL BUSINESS CONCERN 72 KING WILLIAM ROAD, NORTH ADELAIDE, SOUTH AUSTRALIA, AUSTRALIA

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled EARLY DETECTION OF LYSOSOMAL STORAGE DISORDERS by inventor(s)

PETER J MEIKIE, DOUGLAS A BROOKS, JOHN J HOPWOOD

described in

☒ the specification filed herewith

☐ application serial no. _____, filed _____

☐ patent no. _____, issued _____

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME _____

ADDRESS _____

☐ INDIVIDUAL

☐ SMALL BUSINESS CONCERN

☐ NONPROFIT ORGANIZATION

NAME _____

ADDRESS _____

☐ INDIVIDUAL

☐ SMALL BUSINESS CONCERN

☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING STEVEN R HOOD

TITLE OF PERSON IF OTHER THAN OWNER INTELLECTUAL PROPERTY MANAGER

ADDRESS OF PERSON SIGNING 72 KING WILLIAM ROAD

NORTH ADELAIDE SA 5006

SIGNATURE [Signature] DATE 28 OCTOBER 1998

Applicant or Patentee: WOMEN'S AND CHILDREN'S HOSPITAL
Serial or Patent No.: _____
Filed or Issued: _____
Title: EARLY DETECTION OF LYSOSOMAL STORAGE DISORDERS

Attorney's
Docket No.: 2325-1-002

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) & 1.27(b))--INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees to the Patent and Trademark Office regarding the invention entitled EARLY DETECTION OF LYSOSOMAL STORAGE DISORDERS described in:

- ☒ the specification filed herewith.
☐ application serial number _____, filed _____
☐ patent number _____, issued _____

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:*

- ☐ No such person, concern, or organization
☐ Persons, concerns or organizations listed below*

* Note: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME WOMEN'S AND CHILDREN'S HOSPITAL
ADDRESS 72 KING WILLIAM ROAD, NORTH ADELAIDE, SOUTH AUSTRALIA, AUSTRALIA

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION

NAME _____
ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME _____
ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

PETER J MEIKLE
NAME OF INVENTOR
Peter J Meikle
Signature of inventor
23/10/98
Date

DOUGLAS A BROOKS
NAME OF INVENTOR
Douglas A Brooks
Signature of inventor
23/10/98
Date

JOHN J HOPWOOD
NAME OF INVENTOR
John J Hopwood
Signature of inventor
23 Oct 98
Date

NAME OF INVENTOR
Signature of inventor
Date

NAME OF INVENTOR
Signature of inventor
Date

NAME OF INVENTOR
Signature of inventor
Date

EARLY DETECTION OF LYSOSOMAL STORAGE DISORDERS

The present invention relates generally to lysosomal storage disorders and to diagnostic agents for their detection in humans and other animals. More particularly, the present invention is directed to the uses of the LSD markers Lamp-1, Lamp-2, Limp-II, 4-sulphatase, acid phosphatase (ACP), β -hexosaminidase or α -mannosidase, amongst others as diagnostic agents for the detection of many lysosomal storage disorders.

Bibliographic details of the publications referred to in this specification by author are collected at the end of the description.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated element or integer or group of elements or integers, but not the exclusion of any other element or integer or group of elements or integers.

Lysosomal storage disorders (LSD) represent a group of 39 distinct genetic diseases, each one resulting from a deficiency of a particular lysosomal protein or, in a few cases, from non-lysosomal proteins which are involved in lysosomal biogenesis. The importance of these disorders to health care becomes obvious when the group incidence rate for LSD (1:5,000 births) is compared with well known and intensively studied genetic disorders, for which newborn screening is currently performed, such as phenylketonuria (1:14,000) and cystic fibrosis (1:2,500). LSD generally affect young children and have a devastating impact on the child and the family involved. Affected individuals can present with a wide range of clinical symptoms depending upon the specific disorder and the particular genotype involved. Central nervous system dysfunction, from behavioural problems to severe mental retardation, is characteristic of many LSD. In the mucopolysaccharidoses, other symptoms may include skeletal abnormalities, organomegaly, corneal clouding and dysmorphic features (Neufeld and Meunzer, 1995). In severe cases, the child requires constant medical management of the disorder but dies before adolescence.

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Except for those cases with a family history of the disease, pre-symptomatic detection of LSD can only be achieved by newborn screening. Currently, even after the presentation of clinical symptoms, the diagnosis of a LSD is a complex process involving a range of assays performed on urine, blood and in some disorders, skin fibroblasts. These assays are time consuming, expensive and invasive, making them unsuitable for newborn screening applications. In order to justify the screening of the entire neonatal population for a given disorder or group of disorders there are a number of criteria which need to be satisfied, these criteria can be summarised as two broad considerations. Firstly, does neonatal diagnosis provide clear cut benefits to the neonate and family? Secondly, are these benefits reasonably balanced by the total cost of screening?

In recent years, treatment of some LSD has become possible. Cystinosis is treated with cysteamine (Gahl *et al.*, 1987; Markello *et al.*, 1993), a number of LSD including mucopolysaccharidosis (MPS) I and MPS VI have been responsive to bone marrow transplants (Hoogerbrugge *et al.*, 1995; Hopwood *et al.*, 1993) and Gaucher disease is currently being treated by enzyme replacement therapy which, like bone marrow transplantation, is theoretically applicable to a wide range of LSD. Recombinant enzymes deficient in many of LSD have been characterised and there are now numerous animal models which are being used to evaluate enzyme replacement and gene therapies for these disorders. Animal models currently in use include dog models for fucosidosis (Taylor *et al.*, 1989) and MPS VII (Haskins *et al.*, 1992), cat models for MPS I, and VI (Crawley *et al.*, 1996; Haskins *et al.*, 1992), goat models of β -mannosidosis (Jones and Kennedy, 1993) and MPS IIID (Thompson *et al.*, 1992) and mouse models for MPS VII (Sands *et al.*, 1994), galactosialidosis (Zhou *et al.*, 1995) and Niemann-Pick disease (Otterbach and Stoffel, 1995). It is probable that within the next 5 to 10 years effective therapies will be available for many of the LSD.

The effectiveness of these therapies, particularly for those LSD involving central nervous system and bone pathologies, will rely heavily upon the early diagnosis and treatment of the disorder, before the onset of irreversible pathology. Animal studies involving bone marrow transplantation in a fucosidosis dog model, which relates predominantly to central

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nervous system pathology (Taylor *et al.*, 1989) and enzyme replacement therapy studies in an MPS VI cat model (predominantly bone pathology) (Crawley *et al.*, 1996; Crawley *et al.*, 1997) have shown a clear correlation between the age when treatment was commenced and efficacy and that enzyme replacement therapy is effective for the prevention of bone pathology.

5

A further consideration, critical to bone marrow transplant therapy, is that early diagnosis of the LSD will allow clinicians to take advantage of the window of opportunity presented by the naturally suppressed immune system of the neonate to maximise the chances of a successful engraftment.

10

Early detection of these disorders has the added advantage of permitting genetic counselling of the parents, with the option of prenatal diagnosis in subsequent pregnancies, and management of the affected child. Accurate techniques for monitoring progress of the treatment regimes are also required.

15

One common feature of these LSDs is the accumulation and storage of material normally degraded within the lysosome and transported across the lysosomal membrane. It is generally recognised that this results in an increase in the number and size of lysosomes within the cell from approximately 1% to as much as 50% of total cellular volume. However, although the formation of lysosomal storage vacuoles within affected cells is well-known, the process by which lysosomal biogenesis occurs, in particular the nature and role of genes and enzymes which are involved in the process, is poorly understood.

In work leading up the present invention, the inventors sought to identify proteins nucleic acid molecules, oligosaccharides, gangliosides and processes involved in lysosome biogenesis, which are capable of functioning as markers of lysosome storage disorders (hereinafter referred to as "LSD markers"). The LSD markers identified by the inventors have provided for the development of a wide range of diagnostic and therapeutic reagents for the treatment of LSDs in humans and other animals, including the development of procedures to facilitate the presymptomatic detection of all LSDs in a single assay.

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Accordingly, one aspect of the present invention provides a diagnostic method of detecting a lysosomal storage disorder (LSD), monitoring the progress of an LSD or the efficacy of treatment of an LSD in a human or other animal patient comprising assaying the level of expression of an LSD marker as defined herein in a biological sample derived from
5 said patient.

As used herein, the term "LSD marker" or similar term shall be taken to refer to an enzyme, protein, polypeptide or other biomolecule or a homologue, analogue or variant thereof derived from the lysosome of a human or other animal, the presence or level of
10 expression of which is associated with the occurrence, development or onset of at least one LSD in said animal. An LSD marker is usually expressed in a cell derived from a patient having an LSD at a level which is different from that observed for a normal individual.

The present invention extends to the assay of an LSD marker for the diagnosis of a
15 wide range of LSDs selected from, but not limited to the list comprising Pompe disease, Salla disease, Gaucher disease, mucopolysaccharidoses (MPS) including MPS I, MPS II, MPS IIIB, MPS IIIC, MPS IVA, and MPS VI, I-cell disease including ML II/III, Tay-Sach's disease, Fabry's disease, metachromatic leukodystrophy (MLD), Niemann-Pick disease and multiple sulphatase deficiency, amongst others.

20

Those skilled in the art will be aware that a marker may also be used to diagnose a genetic predisposition toward the disease which the marker is used to detect. The present invention therefore extends to the assay of an LSD marker for determining the genetic predisposition of a patient to one or more of the LSD discussed *supra*.

25

An LSD marker according to the present invention may be any lysosomal enzyme, protein, polypeptide or other biomolecule which is up-regulated as a result of the lysosomal proliferation which is characteristic of an LSD or at least accumulates at an increased rate in the lysosomes of patients suffering from an LSD. Those skilled in the relevant art will be
30 aware that the most suitable LSD markers for the present purpose are those enzymes,

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proteins, polypeptides or other biomolecules which are expressed at least 2-fold, preferably at least 5-fold, more preferably at least 10-fold and even more preferably at least 20-fold higher in the cells of LSD-affected patients than in non-affected patients.

5 The present invention extends to the use of any one or more of Lamp-1, Lamp-2, Limp-II, mannose-6-phosphate receptors, 4-sulphatase, acid phosphatase (ACP), β -hexosaminidase, or α -mannosidase, amongst others as an LSD marker.

10 The invention further extends to the use of the foregoing LSD markers in the manufacture of a composition or medicament for the diagnosis and/or treatment of an LSD in a human or animal subject.

15 In a particularly preferred embodiment of the present invention, said LSD marker is the lysosomal Lamp-1 protein. As described in the Examples herein, the inventors have found that the level of Lamp-1, protein is elevated in a wide range of patients suffering from LSDs, when compared to the level of Lamp-1 expression in normal individuals. For example, the level of Lamp-1 protein is 3- to 6-fold higher in the plasma obtained from a patient suffering MPS I compared to a non-affected individual.

20 In one embodiment of the invention, the level of expression of said LSD marker is assayed by measuring the level of enzyme activity of said LSD marker. Several methods are available for the assay of particular enzymes derived from biological samples. Those skilled in the art will be aware that an assay method will vary depending upon the nature of the LSD marker in question, including its substrate preference and co-factor requirement and the tissue
25 or organ from which it was derived. Assay methods for the lysosomal enzymes ACP, β -hexosaminidase, α -L-iduronidase and α -mannosidase are incorporated herein by way of exemplification only.

30 In an alternative embodiment, wherein said LSD marker is either a protein or polypeptide or other stored substrate, the level of expression of said LSD marker may be

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assayed by an immunoassay. Those skilled in the art are aware that, in its broadest context, an "immunoassay" comprises incubating a test sample with one or more immunointeractive molecules specific for said LSD marker, for example an antibody, for a time and under conditions sufficient for binding thereto and detecting said binding. Altered levels of the LSD marker, in particular elevated levels of the LSD marker Lamp-1, compared to the levels detected in non-affected patients, may indicate an LSD.

Conditions for incubating an antibody with a test sample vary, depending upon the format employed in the assay, the detection methods employed and the type and nature of the antibody molecule used in the assay. Those skilled in the art will recognise that any one of the commonly available immunological assay formats, for example radioimmunoassays, enzyme-linked immunosorbent assays (ELISA), diffusion-based Ouchterlony, rocket gel immunoelectrophoresis or *in situ* immunoassays can be readily adapted to the present purpose. Examples of such assay formats can be found in Chard (1986), Bullock (1982, 1983, 1984) or Tijssen (1985). Generally, the assay format will be selected to provide the highest sensitivity of detection for the test sample.

Immunoassays are useful in the quantification of an LSD marker in a test sample, particularly test samples derived from blood samples or isolated cells, in particular to determine whether the level of said LSD marker is elevated compared to normal levels detectable in non-affected individuals. As a consequence, such an immunoassay is of particular use in determining whether a patient may have a lysosomal storage disorder. The invention described herein extends to all such uses of immunointeractive molecules and diagnostic assays which require said immunoassays for their performance.

25

A wide range of immunoassay techniques may be used, such as those described in US Patent Nos. 4,016,043, 4,424,279 and 4,018,653. By way of example only, an antibody raised against the Lamp-1 protein is immobilised onto a solid substrate to form a first complex and a biological test sample from a patient is brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow

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formation of an antibody-Lamp-1 secondary complex, a second Lamp-1 antibody labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing sufficient time for the formation of a tertiary complex of antibody-Lamp-1-labelled antibody. Any unreacted material is washed away, and the presence of the tertiary complex
5 is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal or may be quantitated by comparison with a control sample containing known amounts of hapten. Variations of this assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody, or a reverse assay in which the labelled antibody and
10 sample to be tested are first combined, incubated and then added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, and the possibility of minor variations will be readily apparent. The antibodies used above may be monoclonal or polyclonal.

15 The solid substrate is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs or microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing the
20 molecule to the insoluble carrier.

By "reporter molecule", as used in the present specification, is meant a molecule which, by its chemical nature, produces an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The
25 most commonly used reporter molecule in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes). In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognised, however, a wide variety of different conjugation techniques exist which are readily available to one skilled in the art.
30 Commonly used enzymes include horseradish peroxidase, glucose oxidase, β -galactosidase

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and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. It is also possible to employ fluorogenic substrates, which yield a fluorescent product.

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Alternatively, fluorescent compounds, such as fluorescein, Eu^{3+} or other lanthanide metals, and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state of excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining complex is then exposed to the light of the appropriate wavelength, the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed. It will be readily apparent to the skilled technician how to vary the procedure to suit the required purpose.

20 The immunologically-interactive molecule, in particular an antibody molecule, is also useful in purifying an LSD marker protein or in the manufacture of a compound or medicament for the diagnosis and/or treatment of an LSD in a human or animal subject. Methods for the affinity purification of proteins using antibodies are well-known to those skilled in the art.

25

In a particularly preferred embodiment, the immunoassay employed according to the invention is an ELISA. Antibodies labelled with Eu^{3+} or other lanthanide metals may also be useful as detection molecules in immunoassays based on the time delayed fluorescence, observed with these compounds.

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The immunoassay test samples of the present invention may be derived from any organ, tissue or other biological sample comprising lysosomes. Accordingly, the diagnostic assay of the present invention may be carried out using test samples derived from a human or other animal of any developmental stage including a foetus, embryo, neonate or adult animal, provided that the sample contains a sufficient level of said LSD marker to be detected using a known assay format. Suitable test samples include, but are not limited to crude or partially-purified extracts from cells such as fibroblasts, cultured cell lines, urine, blood and blood-derived products such as serum or plasma, amongst others.

10 The test sample used in the above-described method will vary based upon the assay format, nature of the detection method and the tissues, cells or extracts used as the sample to be assayed. Methods for preparing protein extracts of cells are well-known in the art and can be readily adapted in order to obtain a sample which is suitable for the assay format selected.

15 In a particularly preferred embodiment of the present invention, the test sample is a blood sample or plasma, for example a blood-spot taken from a Guthrie card.

The diagnostic methods described *supra* are useful in antenatal screening for LSD.
20 The invention is also of particular utility in the screening of neonates up to 7 days of age using dried blot spots collected from infants. However, the present invention extends to the use of any assay format or test sample to detect an LSD marker.

A second aspect of the present invention provides a biomolecule to facilitate the
25 detection of a lysosomal storage disorder in a human or other animal, wherein said biomolecule is capable of binding to an LSD marker as defined herein when used in an assay to determine the level of expression of said LSD marker in a biological test sample derived from said human or other animal.

30 According to this aspect of the invention, said biomolecule may be an enzyme

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substrate molecule, a co-factor, an immunologically interactive molecule such as an antibody molecule.

In one embodiment, the biomolecule according to this aspect of the invention is an
5 immunologically interactive molecule.

The term "immunologically interactive molecule" as used herein shall be taken to refer to a polyclonal or monoclonal antibody or a functional derivative thereof, for example a Fab, SCAB (single-chain antibody) or an antibody conjugated to an enzyme, radioactive,
10 paramagnetic or fluorescent tag, the only requirement being that said immunologically interactive molecule is capable of binding to an LSD marker or a derivative, part, fragment, analogue or homologue thereof.

Preferably, the immunologically interactive molecule is in the form of an antibody
15 such as a polyclonal or monoclonal antibody. The present invention extends to immunologically interactive fragments, parts, derivatives, homologues or analogues of these antibodies. Such antibodies may be in an isolated or purified form comprising at least 25% (w/w), more preferably at least 50% (w/w), even more preferably at least 60-75% (w/w) and even still more preferably at least 80-95% (w/w) of immunoglobulin on a protein basis.
20 Alternatively, the antibodies may be present in the form of isolated hybridoma, culture supernatant, tissue extract, serum or whole blood or ascites fluid.

Conventional methods can be used to prepare the immunologically interactive molecules. By using a polypeptide comprising all or a fragment of an LSD marker as defined
25 herein, polyclonal antisera or monoclonal antibodies can be made using standard methods. For example, any mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of an antigen comprising an LSD marker to elicit an antibody response in the mammal. Techniques for conferring immunogenicity on an antigen include conjugation to carriers or other techniques well known in the art. For example, the antigen can be
30 administered in the presence of adjuvant. The progress of immunization can be monitored

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by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired IgG molecules corresponding to the polyclonal antibodies may be isolated from the sera.

5

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art. For example, the hybridoma technique originally developed by Kohler and Milstein (1975) as well as other techniques such as the human B-cell hybridoma technique (Kozbor *et al.*, 1983), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, 1985), and screening of combinatorial antibody libraries (Huse *et al.*, 1989). Hybridoma cells can be screened immunochemically for production of antibodies which are specifically reactive with the antigen and monoclonal antibodies isolated.

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As with all immunogenic compositions for eliciting antibodies, the immunogenically effective amounts of the polypeptides of the invention must be determined empirically. Factors to be considered include the immunogenicity of the native antigen, whether or not the antigen will be complexed with or covalently attached to an adjuvant or carrier protein or other carrier and route of administration for the composition, i.e. intravenous, intramuscular, subcutaneous, *etc.*, and the number of immunizing doses to be administered. Such factors are known in the art and it is well within the skill of immunologists to make such determinations without undue experimentation.

25 The term "antibody" as used herein, is intended to include fragments thereof which are also specifically reactive with a polypeptide which comprises, mimics, or cross-reacts with a B cell or T cell epitope of an LSD marker according to the embodiments described herein, in particular the Lamp-1 and 4-sulphatase. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab')₂ fragments can be generated by

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treating antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

It is within the scope of this invention to include any second antibodies (monoclonal, polyclonal or fragments of antibodies) directed to the first mentioned antibodies discussed above. Both the first and second antibodies may be used in detection assays or a first antibody may be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any region of a polypeptide which comprises, mimics, or cross-reacts with a B cell or T cell epitope of an LSD marker as hereinbefore defined.

The polyclonal, monoclonal or chimeric monoclonal antibodies can be used to detect and/or quantify an LSD marker or a derivative, homologue or analogue thereof in various biological materials, for example they can be used in an ELISA, radioimmunoassay or histochemical tests. Thus, the antibodies can be used to test for binding to an LSD marker or a derivative, homologue or analogue thereof in a sample or to determine B cell or T cell epitopes of an LSD marker.

Accordingly, in another embodiment of the present invention, the above-described antibodies are detectably labeled to facilitate their use in immunoassays conducted either *in vitro*, *in vivo* or *in situ*. Antibodies can be detectably labeled through the use of radioisotopes, affinity labels such as biotin, avidin and the like, enzyme labels such as horse radish peroxidase, alkaline phosphatase and the like, fluorescent labels such as FITC, rhodamine and the like or Eu³⁺, using paramagnetic atoms, amongst others. Procedures for accomplishing such labeling are well-known in the art.

Preferably, the antibody is raised against a protein or polypeptide. However, in a particularly preferred embodiment, the present invention provides antibodies which are capable of recognising either the Lamp-1, Lamp-2, Limp-II or 4-sulphatase protein as described herein.

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A further aspect of the invention contemplates a diagnostic kit for determining the level of expression of an LSD marker in a biological sample derived from a human or other animal suspected of having a lysosome storage disorder.

5 In a preferred embodiment of this aspect of the invention, said kit comprises in a first compartment several first containers adapted to contain an LSD marker enzyme, protein, polypeptide or a derivative, homologue or analogue thereof in recombinant or synthetic form and optionally adsorbed thereto, and several second containers adapted to contain an antibody which recognises said LSD marker or a B cell or T cell epitope thereof, wherein said
10 antibody is optionally labelled with a reporter molecule capable of producing a detectable signal as hereinbefore described. There are also provided several third containers which contain a second antibody which recognises the first antibody and is optionally conjugated to a reporter molecule. If the reporter molecule is an enzyme, then several fourth containers are provided which contain a substrate molecule for said enzyme to facilitate detection of the
15 enzyme:antibody:LSD marker complex or alternatively, the enzyme:antibody:antibody:LSD marker complex where a second antibody has been used. The reporter molecule used in this kit may also be a radio-isotope, a fluorescent molecule, or bioluminescent molecule, amongst others.

20 Optionally, the kit may further be contained in a package which comprises microtitre wells in one section, in which reactions may be performed. Accordingly, in one embodiment, the microtitre wells may be the equivalent of the first compartment hereinbefore described and contain the LSD marker or a derivative, homologue or analogue thereof, adsorbed thereto.

25

Optionally, the first, second, third and fourth containers of said kit may further be colour-coded for ease-of use.

In an exemplified use of the subject kit, the contents of the first container may be
30 bound to a microtitre well contained in the package, if not provided in a format where said

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contents are already adsorbed to said microtitre well, and a biological sample to be tested is added and incubated for a time and under conditions sufficient for an antigen-antibody complex to form in said microtitre well. Following a washing step to remove unbound antibodies and other unbound protein, the contents of the third container are added to the antigen-antibody complex contained in the microtitre well and the reaction allowed to proceed for a time, and under conditions sufficient to allow the formation of the tertiary antigen-antibody-antibody complex. A control reaction may be performed in which the contents of the second container are added to the contents of the first container for a time and under conditions suitable for the formation of an antigen-antibody complex. If the antibody of the second container is not labelled with a reporter molecule, then the contents of the third container may be added for a time and under conditions suitable for the formation of a tertiary antigen-antibody-antibody complex to form. The tertiary antigen-antibody-antibody complexes of the control reaction and the test sample are then subjected to a detecting means. Alternatively, if the contents of the second container are labelled with a reporter molecule the antigen-antibody complex of the control reaction may be subjected directly to a detecting means. The means of detection of a secondary antigen-antibody or a tertiary antigen-antibody-antibody complex are numerous, as hereinbefore described and will be known to those skilled in the art. Where said means is an enzyme reaction, the contents of the fourth container are added to said secondary or tertiary complex thus formed for a time and under conditions suitable to enable the enzyme reaction to occur.

In analysing the results obtained using the subject kit, the amount of LSD marker contained in the control reaction is predetermined to provide a result which is consistent with the result obtained for a normal non-affected patient and therefore the control reaction provides a basis for comparison with the test sample. A signal obtained for the test sample which is higher than that of the control indicates a higher level of the subject LSD marker being tested. Such a result may indicate that the patient is suffering from a lysosomal storage disorder.

The present invention further extends to any kit comprising a biomolecule which is

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capable of detecting a lysosomal storage disorder in a human or other animal, wherein said kit is in a form which is suitable for an assay to detect expression of an LSD marker as hereinbefore defined. The present invention also extends to kits comprising multiple of said biomolecules to facilitate the detection of more than one LSD marker.

5

For the purposes of exemplification only, the present invention is further described by the following Figures and Examples.

In the Figures:

10

Figure 1 is a graphical representation showing the cross-reactivity of polyclonal antisera raised against immunopurified LAMP-1. Microtitre wells were coated with polyclonal antibody at a level of either 5 $\mu\text{g/ml}$ (○) or 10 $\mu\text{g/ml}$ (□) using the one-step quantification method as described in Example 3.

15

Figure 2 is a graphical representation showing the cross-reactivity of Eu^{3+} - labelled antibody to LAMP-1. Standard solutions of LAMP-1 containing either 10 ng/ml (●), 20 ng/ml (□) or 40 ng/ml (○) were prepared and the fluorescent response for each sample was determined at a range of Eu^{3+} labeled antibody concentrations in the time delayed fluorescence immunoassay, using the one-step quantification method as described in Example 3.

20

Figure 3 is a graphical representation showing LAMP-1 levels in blood spots from 186 newborns, as determined using the one-step quantification method described in Example 3. The x-axis indicates the level of LAMP-1 (ng/blood spot). The number of individuals within each range of LAMP-1 levels is indicated on the y-axis.

25

Figure 4 is a graphical representation showing LAMP-1 levels in plasma from 320 LSD affected individuals, representing 25 lysosomal storage disorders, and 152 unaffected individuals. LAMP-1 levels were determined using 5-10 μl samples in the two-step quantification method described in Example 3. Centre bars show the median LAMP-1 level

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for each disorder, shaded area shows the 25th and 75th percentiles and the upper and lower bars show the limits of the range. Outliers (○) and extreme outliers (*) are also indicated. GM1=GM1 gangliosidosis; MLD=metachromatic leukodystrophy; MSD= multiple sulphatase deficiency; N-P=Niemann-Pick; SAS=sialic acid storage; TSD=Tay Sachs disease.

Figure 5 is a graphical representation showing the LAMP-2 levels in plasma samples derived from 14 LSD affected and 4 (Normal) unaffected individuals. The status of individuals is indicated on the x-axis.

10

Figure 6 is a photographic representation of electron and immunofluorescence micrographs of fibroblasts from the Sucrosome Model. Panels A, B, C, & D are electron micrographs of fibroblasts grown in media containing 100 mM sucrose for (A) 0 days; (B) 1 days; (C) 7 days; (D) 28 days. Panels E, F, G, & H are immunofluorescence micrographs of fibroblasts grown in media containing 100 mM sucrose for (E) 0 days; (F) 1 day; (G) 7 days; (H) 28 days. Immunofluorescence micrographs were photographically exposed for equivalent times (45 sec) to enable comparison of relative intensities. Scale bar is 4 μ for EM micrographs and 20 μ for immunofluorescence micrographs.

Figure 7 is a graphical representation showing the relative LAMP-1 protein levels in the Sucrosome Model. Skin fibroblasts were grown in the presence or absence of 100 mM sucrose for up to 28 days. Cells were harvested at set time points and the LAMP-1 determined in cells grown in the presence (■) and absence (□) of sucrose. LAMP-1 values were normalized to total protein content.

25

Figure 8 is a photographic representation of electron micrographs showing the correction of storage in the Sucrosome Model. Electron micrographs of fibroblasts grown on normal media (Panel A) or sucrose containing media (Panels B-H). Cells were grown for 14 days and either harvested immediately (Panels A and B), switched onto BME media and harvested after 1 day (Panel C); 3 days (Panel E) and 7 days (Panel G) or switched onto media containing

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invertase and harvested after 1 day (Panel D); 3 days (Panel F) and 7 days (Panel H). Scale bar is 2μ .

Figure 9 is a graphical representation showing the relative levels of LAMP-1 protein after correction of storage on the Sucrosome Model. Skin fibroblasts were grown in the presence of sucrose for 14 days to accumulate storage vacuoles. The cells either continued to grow in media containing sucrose or were corrected by switching the cells to BME media or to BME media containing invertase for up to a further 21 days. LAMP-1 was determined at time points through the 21 day period and plotted. Cells were grown on sucrose containing media only (■), or on sucrose containing media for 14 days then placed on BME media (▲), or on sucrose containing media for 14 days then placed on invertase containing media (●), or on BME media throughout the time-course (□).

Figure 10 is a photographic representation of immunofluorescence micrographs showing fibroblasts from affected cell-lines labelled using anti-LAMP-1 monoclonal antibody. Fibroblasts were from Pompe (Panel A), Salla (Panel B), MPS-II (Panel C), and MPS-VI (Panel D) affected cell-lines. Micrographs were photographically exposed for equivalent times (45 sec) to enable comparison of relative intensities. Scale bar is 20μ .

Figure 11 is a photographic representation of electron micrographs showing correction of storage in patient cell-lines. Cell lines were from a Pompe affected cell (Panel A); Pompe corrected cell (Panel B); MPS-II affected cell (Panel C); MPS-II corrected cell (Panel D); MPS-VI affected cell (Panel E); MPS-VI corrected cell (Panel F). Electron microscopy confirmed correction in Pompe (Panels A & B), MPS-II (Panels C & D) and MPS-VI (Panels E & F) affected cells, with the size of storage vacuoles dramatically decreasing (Panels B,D,F) within 7 days after the addition of the enzyme. Scale bar is 2μ .

Figure 12 is a graphical representation showing relative LAMP-1 levels in Pompe-affected fibroblasts and corrected Pompe fibroblasts. Pompe affected fibroblasts and normal skin fibroblasts were grown to confluency then either continued on normal media or corrected by

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switching the cells to media containing α -glucosidase for up to a further 14 days. Relative LAMP-1 levels were determined at time points through the second 14 day period and plotted. Normal cells are represented by (\blacktriangle), Pompe affected cells on BME media by (\blacksquare) and Pompe affected cells on α -glucosidase containing media by (\square).

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EXAMPLE 1**Production of Antibodies Against LAMP-1.**

Anti-LAMP-1 monoclonal antibody (clone BB6) and anti-LAMP-1 polyclonal antibody
5 have been described previously and are reasonably available to the public (Carlsson and Fukuda,
1989; Dahlgren *et al.* 1995). For the production of the anti-LAMP-1 monoclonal antibody clone
4F5, mice were immunised with lysosomal membranes purified from human placenta (Meikle
et al., 1995). Membranes were denatured by boiling in 1% (v/v) 2-mercaptoethanol for 5 min
and the pelleted membranes extracted with chloroform/methanol (2:1) x 2. Female Balb/C mice
10 were immunised according to the following schedule. 50 µg antigen in 400 µl PBS by intra-
splenic injection. 14 days later 50 µg antigen in 200 µl of PBS/incomplete Freund's adjuvant
emulsion by intraperitoneal injection, 21 days later 50 µg antigen in 200 µl PBS by
intraperitoneal injection. Four days later the spleen cells were harvested and fused with P3.653
myeloma cells as described by Zola and Brooks (1982).

15

EXAMPLE 2**Purification of LAMP-1**

20 Total membranes from human placenta were prepared as follows, fresh placenta (450
g) was dissected into 1-2 cm strips and washed three times with cold 0.25 M sucrose, 1 mM
EDTA, pH 7.0 then minced and homogenised (Omnimix 1 min, full speed) in 800 ml of the
same buffer. The cell debris were pelleted at 750 x g for 10 min and homogenised a further two
times. The supernatants were combined, filtered through cotton gauze and made up to 10 mM
25 CaCl₂. After 1 h at 4°C the placental membranes were pelleted at 10,000 x g for 90 min. The
membranes were resuspended in 1 M NaCl (320 ml) freeze/thawed 3 times then pelleted at
100,000 x g for 1 h. The 1 M NaCl wash was repeated and the membranes finally taken up into
320 ml of solubilisation buffer (50 mM MOPS, 1 mM EDTA, 150 mM NaCl, 10%(v/v)
glycerol, 1%(w/v) Thesit, pH 7.0) and stirred at 4°C for 16 h. The insoluble material was
30 pelleted at 100,000 x g for 1 h and the supernatant recovered.

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The supernatant was made up to 3 mM CaCl_2 , 3 mM MgCl_2 then applied to a 70 ml column of concanavalin A Sepharose (Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated in solubilisation buffer containing the CaCl_2 and MgCl_2 . The column was washed with the same buffer and the bound proteins including LAMP-1 were eluted by solubilisation buffer containing 10% (w/v) α -methyl mannoside. The eluate was applied to a 5 ml column of Red Dye No. 78 (Centre for Protein and Enzyme Technology, LaTrobe University, Bundoora Australia) and the LAMP-1 recovered in the flow through.

Anti-LAMP-1 monoclonal antibody 4F5 (20 mg) was coupled to Affigel (10 ml) and used for the affinity purification of LAMP-1. The Red Dye flow through (120 ml) was mixed with the anti-LAMP-1 affinity gel and rocked gently for 16 h at 4°C. the gel was then poured into a column and washed with PBS. The LAMP-1 was eluted from the column with 100 mM triethylamine, pH 11.5, dialysed against water and lyophilised.

The preparation of total placental membranes resulted in a LAMP-1 yield of only 25% of the total LAMP-1 present in the placenta, despite the fact that greater than 60% of the lysosomal membrane enzyme acetyl coenzyme A: α -glucosaminide N-acetyltransferase was associated with these membranes.

However, solubilisation of the membranes, chromatography on concanavalin A Sepharose and subsequent red dye chromatography all gave greater than 80% recovery of LAMP-1. From a single placenta, approximately 1 mg of LAMP-1 was recovered in the red dye column flow through.

The successive immunoprecipitation of the LAMP-1 from this sample resulted in the recovery of approximately 100 μg per precipitation.

Purified LAMP-1 appeared as a homogeneous band on coomassie stained SDS-PAGE (not shown), was quantified by the bicinchoninic acid method and subsequently used as a standard for the immunoquantification of LAMP-1 protein.

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EXAMPLE 3**Immunoquantification of LAMP-1.**

Methods: Determination of LAMP-1 was performed using a Time Resolved
5 Fluorescence Immunoassay (TRFIA). In this assay, the detecting antibody is labelled with a
lanthanide metal (usually europium) chelated into N¹-(p-isothiocyanatobenzyl)-
diethylenetriamine-N¹, N², N³, N³-tetraacetic acid. Detection of the labelled antibody is
achieved by lowering the pH to release the Eu³⁺ from the antibody and the subsequent complex
formation with 2-naphthyltrifluoroacetone and tri-n-octylphosphine oxide. The complex formed
10 is highly fluorescent with a relatively long half-life which enables the use of time resolved
fluorescence detection to eliminate background interferences (Diamandis, 1988; Hemmila,
1988).

Anti-LAMP-1 monoclonal antibody (clone BB6) was labeled with europium using the
15 DELFIA[®] Eu³⁺-labeling kit (Wallac Inc. North Ryde, Australia). The labeled antibody was
purified from aggregated antibody and free Eu³⁺ label on a Pharmacia Superose 12 Fast Phase
Liquid Chromatography column (1.5 x 30 cm) eluting with 50 mM TRIS/HCl, pH 7.8, 0.9%
(w/v) NaCl. The level of Eu³⁺ conjugated to each antibody molecule was determined from
protein and fluorescence levels of the conjugate.

20

Samples were assayed for LAMP-1 by either a one-step or two-step method.

In the one-step method, microtitre plates (Immulon 4 Dynatech Laboratories, Inc.
Virginia, USA) were coated with anti-LAMP-1 polyclonal antibody at 5 µg/ml for 4 h at 37°C
25 (100µl/well diluted in 0.1 M NaHCO₃) and washed with DELFIA[®] wash buffer (x 6). Samples
were diluted in DELFIA[®] assay buffer containing 200 ng/ml of Eu³⁺ labeled anti-LAMP-1
monoclonal antibody (100 µl/well) and incubated in wells overnight at 4°C. Plates were
incubated at room temperature for 1h then washed (x 6). DELFIA[®] enhancement buffer (200
µl per well) was added, the plates shaken for 10 min at room temperature and the fluorescence
30 measured on a 1234 DELFIA[®] Research Fluorometer.

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In the two-step method, if samples contained chemicals which were incompatible with the Eu^{3+} label (eg. EDTA, citrate), the plates were coated with the polyclonal antibody and washed as described *supra*; samples were diluted in DELFIA® assay buffer without the Eu^{3+} labeled antibody and incubated overnight at 4°C, incubated at room temperature for 1h and washed (x 6). Assay Buffer containing 200 ng/ml of Eu^{3+} labeled anti-LAMP-1 monoclonal antibody (100µl) was added to each well and incubated at room temperature for 2h. Plates were then washed (x 6), enhancement buffer was added and the fluorescence measured as described.

For determination of LAMP-1 in blood spots, the one-step method was used with the following modifications:

Blood spots were incubated with 200 µl of assay buffer containing 200 ng/ml of Eu^{3+} labeled anti-LAMP-1 monoclonal antibody. The plates were shaken for 1 h at room temperature prior to the overnight incubation at 4°C, then again for 1 h at room temperature prior to washing and addition of enhancement buffer.

Results: Labeling of the BB6 monoclonal antibody with Eu^{3+} resulted in approximately 5 Eu^{3+} atoms per antibody molecule. When used in the one-step immunoquantification assay as described, this gave a linear response over the range 0.1 ng -12.5 ng/well LAMP-1. A lower response was obtained with plates coated with 5 µg/ml polyclonal antibody as compared to 10 µg/ml (Fig. 1). The two-step assay gave approximately 50% of the signal of the one-step assay with a linear range up to 25 ng/well. A linear response was also observed when whole blood or plasma from either unaffected or LSD affected individuals was assayed (1-50 µl). The intra-assay coefficient of variation was less than 9%. The inter-assay variation, as determined from the coefficients of variation of the standard curve points over 9 assays performed on 4 different days, ranged from 2% to 9% across the linear range of the assay. The standard curves were subject to linear regression analysis and gave values for the standard error of estimate of between 0.14 and 0.45 ng/well with an average of 0.26 ng/well, the intercept values had an average of 0.08 ng/well with a standard deviation of 0.16 ng/well.

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Precision studies were also performed on plasma samples. Five plasma samples ranging in LAMP-1 concentration from 300 to 1200 ng/ml were assayed in triplicate on 10 separate occasions, the intra-assay variation was less than 6% while the inter-assay variation was less than 8%.

5

Analytical recovery studies were performed by adding a known amount of purified LAMP-1 to varying amounts of plasma in the 2-step assay, the results showed an inverse relationship between the level of plasma and the recovery of exogenously added LAMP-1. When 10 ml of plasma was included per well (the largest volume assayed) recovery of 10 exogenous LAMP-1 was 68%.

The effect of the concentration of Eu^{3+} labeled antibody on the assay was also investigated. Data presented in Figure 2 indicate that the increase in signal showed an almost linear correlation with antibody concentration up to 400 ng/ml.

15

In all experimental assays performed, 200 ng/ml of labeled antibody resulted in suitable sensitivity.

EXAMPLE 4

20

LAMP-1 Levels in Blood

Patient Samples: Blood spots used in this study were part of the routine samples collected from neonates from the South Australian population. Plasma samples used were from samples submitted to the National Referral Laboratory for LSD screening and samples 25 processed for routine biochemistry.

Blood Spots: To determine the suitability of LAMP-1 as a marker for newborn screening for LSD, we immunoquantified the LAMP-1 present in blood spot samples taken from 186 unaffected newborns. We observed a characteristic skewed distribution with a median of 1.3 ng/spot and the 5th and 95th percentiles at 0.76 and 3.3 ng/spot respectively (Fig. 3). There 30 was no correlation between LAMP-1 level and age, sex or birth weight of the newborns.

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Plasma: To investigate the levels of LAMP-1 in LSD affected individuals we retrieved plasma samples from the Departmental archives of 320 LSD affected individuals, representing 25 disorders and 152 age matched (median=7, range=0-66) unaffected individuals. LAMP-1 levels in these samples (Fig. 4) showed a tight distribution in the normal population with a median of 303 ng/ml and the 5th and 95th percentiles at 175 and 448 ng/ml respectively. The majority of the LSD affected individuals had LAMP-1 levels which were above the normal range (72% above the 95th percentile of the control population), with some individuals having up to 10 times the median level of the control population.

When analysed by individual disorder, 16 of the 25 disorder groups tested were observed to have greater than 88% of individuals above the 95th percentile of the control population, with 12 groups having 100% above the 95th percentile (Table 1).

A significant correlation was observed between LAMP-1 levels and age in the normal population, with a Pearson correlation coefficient of -0.37 and a significance level less than 0.001. Of the affected groups, only Fabry and Gaucher had sufficient numbers and age range to test for a correlation. The Fabry group showed a Pearson correlation coefficient of -0.49 with a significance level of 0.02, whereas the Gaucher group showed no significant correlation between LAMP-1 levels and age.

20

Whole Blood: Samples of whole blood from six unaffected individuals were fractionated and the proportion of LAMP-1 present in white cells, red cells, and plasma was determined. Peripheral blood leucocytes and plasma were isolated from whole blood collected in heparinised tubes by the method of Kampine *et al.* (1967) and the white cell pellet was resuspended in saline containing 1% (v/v) Nonidet P-40 (lysis buffer). Red cells isolated in the same procedure were washed twice with saline before being resuspended in lysis buffer. The saline washes were centrifuged to pellet the white cells which were combined with the original white cell pellet, the supernatants were pooled with the plasma for determination of LAMP-1 protein.

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Whole blood samples had an average of 226 ± 31 ng/ml of LAMP-1, with the distribution of LAMP-1 being $53 \pm 7\%$ in the plasma, $32 \pm 5\%$ in the red blood cell pellet and $15 \pm 5\%$ in the white cell pellet.

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TABLE 1
Detection of Lysosomal Storage Disorders Based on
Elevated LAMP-1 Levels in Plasma

5	Disorder	Stored Substrates	N= ^a	Age ^b	Median ^c	%>95 ^d
	Control		152	7 (0-66)	308	5
	Fabry	α -galactosylsphingolipids: oligosaccharides	24	27 (4-47)	359	25
	Galactosialidosis	oligosaccharides	1	16	1653	100
10	Gaucher	glucoceramide	51	12 (0-68)	956	92
	GM1-gangliosidosis	GM1-gangliosides: oligosaccharides, glycolipids: keratan sulphate	12	1 (0-15)	1071	92
	I-cell	glycolipids: oligosaccharides	15	3 (0-25)	1815	100
	Krabbe	galactoceramides	12	0.4 (0-1)	407	17
	α -Mannosidosis	α -mannosides	4	4 (3-15)	1181	100
15	Metachromatic Leukodystrophy	sulphatides	32	3 (0-30)	379	19
	MPS ^e I	dermatan sulphate: heparan sulphate	22	1 (0-29)	1470	100
	MPS II	dermatan sulphate: heparan sulphate	23	3 (0-11)	1406	100
	MPS IIIA	heparan sulphate	20	4 (1-17)	1039	100
	MPS IIIB	heparan sulphate	16	3 (2-21)	880	100
20	MPS IIIC	heparan sulphate	3	11 (6-20)	787	100
	MPS IIID	heparan sulphate	3	3 (0-3)	1010	100
	MPS IVA	keratan sulphate	16	3 (0-12)	699	88
	MPS VI	dermatan sulphate	12	4 (0-16)	1018	92
	Multiple sulphatase deficiency	sulphatides: glycolipids: GAG ^f	2	5 (3-7)	507	100
25	Niemann-Pick (A and B)	sphingomyelin	9	22 (1-44)	385	33
	Niemann-Pick (C)	cholesterol: sphingomyelin	10	12 (0-41)	391	20
	Pompe	glycogen	4	0.4 (0-1)	462	25
	Sandhoff	GM2-gangliosides: oligosaccharides	6	1 (1)	565	100
	Sialic Acid Storage	sialic acid: glucuronic acid	2	2 (0-3)	1035	100
30	Tay-Sachs (I)	GM2-gangliosides: oligosaccharides	17	1 (0-27)	425	41
	Tay-Sachs (AB)	GM2-gangliosides	2	7 (6-8)	489	100
	Wolman	cholesterol esters	2	1 (0-1)	496	50

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- a Number of patient samples in each group.
- b Median age of patients in each group, bracketed numbers indicate the range of ages of patients in each group.
- c Median value of Plasma LAMP-1 expressed as ng/ml.
- 5 d The percentage of each disorder group which had LAMP-1 levels above the 95th percentile of the control population (448 ng/ml).
- d Mucopolysaccharidosis.
- e Glycosaminoglycan.

448 ng/ml

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EXAMPLE 5**LAMP-1 levels in LSD Patients Following Treatment**

Plasma levels of LAMP-1 were determined as described in Example 3, for plasma samples derived from LSD affected patients before and after treatment comprising bone marrow transplantation (for MPS I and MPS VI) or enzyme replacement therapy (for Gaucher disease). The results show that in each case the plasma LAMP-1 levels decrease, to within or close to the normal range, following treatment (Table 2). These data suggest that LAMP-1 may be useful as a marker of the progression and efficacy of therapy.

TABLE 2**LAMP-1 levels in LSD Patients Following Treatment**

	Patient Number	Disorder	Age	LAMP-1 Level^a
	1	MPS I	1.8	1210
15	1	MPS I	2.3	1135
	BMT ^b		2.3	
	1	MPS I	2.8	504
	1	MPS I	8.0	630
20	2	MPS VI	1.5	1115
	2	MPS VI	12.0	734
	BMT		12.0	
	2	MPS VI	12.1	474
	2	MPS VI	12.2	457
25	3	Gaucher	5.8	1268
	ERT ^c		11.5	
	3	Gaucher	12.5	744
	3	Gaucher	12.8	602
30	3	Gaucher	12.9	616

^a LAMP-1 level present in plasma (ng/ml)^b Bone Marrow Transplant^c Enzyme Replacement Therapy

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EXAMPLE 6**Levels of Plasma LAMP-1 in Gaucher Affected Siblings**

LAMP-1 was determined in the plasma of two Gaucher affected siblings. Both
5 individuals were genotyped to have the same mutations, yet one was asymptomatic at age 7,
while the other showed a severe clinically phenotype at age 4. The asymptomatic sibling had
a LAMP-1 level of 417 ng/ml (within the normal range), the affected sibling had a LAMP-1
level of 787 ng/ml (above the normal range)

10 These data suggest that LAMP-1 is a useful indicator of clinical severity and the
requirement for therapy and the effectiveness of therapy

EXAMPLE 7**15 Immunoquantification of LAMP-2**

Monoclonal and polyclonal antibodies against LAMP-2 and pure LAMP-2 were
supplied by Dr. S. Carlsson. Department of Medical Biochemistry and Biophysics, Umea
University, Umea, S-901 87, SWEDEN.

20

The immunoquantification of LAMP-2 was essentially as described for LAMP-1 except
that a secondary, Eu³⁺ labelled, rabbit anti-mouse IgG was used in place of an anti-LAMP-2
monoclonal antibody label directly with Eu³⁺.

25 Microtitre plates (Immulon 4 Dynatech Laboratories, Inc. Virginia, USA) were coated
with anti-LAMP-2 polyclonal antibody at 5 µg/ml for 4 h at 37°C (100µL/well diluted in 0.1
M NaHCO₃) and washed with DELFIA® wash buffer (x 6). Samples were diluted in DELFIA®
assay buffer containing 1000 ng/ml of anti-LAMP-2 monoclonal antibody (100 µL/well) and
incubated in wells overnight at 4°C. Plates were then washed (x 6) and incubated with Eu³⁺
30 labelled, rabbit anti-mouse IgG DELFIA® for 2 h at 20°C. The plates were washed (x6) and

- 30 -

enhancement buffer (200 μ L per well) was added, the plates shaken for 10 min at room temperature and the fluorescence measured on a 1234 DELFIA® Research Fluorometer.

LAMP-2 was determined in plasma samples from 14 LSD affected individuals and 4 unaffected individuals. The unaffected individuals had a mean level of 3.1 ug/ml, the levels of the LSD affected individuals ranged from 1.2 to 17.7 ug/ml (Fig 5). Elevation of LAMP-2 was seen in all disorders where an elevation of LAMP-1 had been observed and in addition, a Pompe affected individual which did not show an elevation of LAMP-1 did show an elevation of LAMP-2. The level of LAMP-2 in plasma was approximately 10-fold higher than the level of LAMP-1 which will enable the easier detection of LAMP-2 in blood samples.

EXAMPLE 8

Sucrosome Formation as a Model of Lysosomal Storage Disorders

15

Cell Culture: Human diploid fibroblasts were established from skin biopsies submitted to Women's and Children's Hospital, Adelaide, Australia, for diagnosis (Hopwood *et al.*, 1982). Cell lines were maintained according to established procedures in Basal medium Eagle's (BME), 10% (v/v) fetal calf serum (FCS) and antibiotics, in a 5% (v/v) CO₂ atmosphere incubator, unless otherwise stated.

Skin fibroblasts were plated in 75cm² flasks with BME media and allowed to reach confluence. Once confluent, cells were grown in the BME media containing 100 mM sucrose for 1-35 days to induce sucrosome formation, while control cells remained on BME (no sucrose). LSD affected cell lines were grown under the same conditions, without sucrose. For electron microscopy, fibroblasts were grown in 25cm² tissue culture flasks under identical conditions.

Sucrosome containing cells were corrected by the addition of invertase (0.1 mg/ml, Grade VII, ~400 U/mg; Sigma) to the BME culture media, or alternatively, the cells were placed

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on sucrose free BME media. LSD affected cell lines were corrected by the addition of α -glucosidase (50 nmol/min/ml), as described in Fuller *et al.* (1995), for Pompe disease cells, iduronate-2-sulphatase (50 nmol/min/ml) (Bielicki *et al.*, 1993) for MPS-II or 4-sulphatase (0.5 μ g/ml) (Anson *et al.*, 1992) for MPS-VI.

5

Preparation of Cell Extracts: Cells were washed twice with Dulbecco's phosphate-buffered saline (PBS) and removed from the flask by trypsination (2 ml trypsin-versene solution (CSL Limited, Melbourne, Australia) per flask for 5 min at 37°C). The cells were immediately washed twice with cold PBS and cells from one flask from each time point were resuspended
10 in 200 μ L saline containing 1% (v/v) Nonidet P40. Cell lysates were prepared by 5 cycles of freeze/thaw, clarified by microcentrifugation (1000 x g, 5 min) and assayed for lysosomal enzymes and proteins.

Protein/Enzyme Assays: Protein was determined using the bicinchoninic acid method
15 with bovine serum albumin (BSA) as a standard (Smith *et al.*, 1985). The following enzyme activities were determined using fluorogenic substrates. Acid phosphatase activity was determined with 4-methylumbelliferyl-phosphate (Kolodny and Mumford, 1976), β -hexosaminidase with 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside (Leaback and Walker, 1961), α -mannosidase with 4-methylumbelliferyl- α -L-mannopyranoside
20 (Avila and Convit, 1973) and α -iduronidase with 4-methylumbelliferyl- α -L-iduronide (Clements *et al.*, 1985). The activity of 4-sulphatase was determined using the immune-capture assay as described by Brooks *et al.* (1994). α -Ketoglutarate dehydrogenase was measured by a radiochemical assay in which α -[1- 14 C]ketoglutaric acid was generated in situ from [1- 14 C]glutamate, as described by Singh *et al.* (1987). Galactosyltransferase was measured by a
25 modification of Rome *et al.* (1979), where 3 H-UTP-galactose was used in place of 14 C-UTP-galactose. Enzyme activity levels were normalized to total protein content.

Immunoquantification of LAMP-1: Lamp-1 determinations were performed as described in Example 3.

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Electron Microscopy: Fibroblast cells were harvested then fixed for 2-3 hours with 2% (v/v) formaldehyde and 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer containing 5 mM calcium chloride, pH 7.2. Specimens were postfixed in 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer and 5 mM calcium chloride, pH 7.2. Specimens were dehydrated in a graded series of aqueous ethanol and embedded in Spurr's low viscosity epoxy resin (TAAB, Berkshire, United Kingdom).

Semi-thin (1µm thick) survey sections were obtained using an Ultracut Ultramicrotome (Leica, Vienna, Austria) and stained with 1% (w/v) toluidine blue in 1% (w/v) borax. For each block a correctly orientated area for sectioning was selected. Ultrathin sections with a silver interference colour (60-90 nm thick) were cut and mounted on 100 mesh hexagonal copper (G 100 HEX) grids (Gilder Grids, Grantham, United Kingdom). Sections were stained with 2% (w/v) uranyl acetate in 50% (v/v) aqueous ethanol followed by Reynolds lead citrate and examined with a Hitachi H-7000 transmission electron microscope (Hitachi, Tokyo, Japan), operating at an accelerating voltage of 75 kV.

Immunofluorescence: After harvesting, cells for immunofluorescence (~ 10⁵ cells) were plated and grown for a further 24 hr at 37°C in tissue culture chamber slides (Nunc) in media plus or minus sucrose or in media containing 0.1 mg/ml invertase as indicated. Cells were then washed twice with PBS and fixed with 1% (v/v) formaldehyde in PBS for 30 min at 4°C, washed twice with methanol and allowed to air-dry. Slides were stored at -20°C until further processing.

Slides were allowed to come to room temperature, and washed twice with PBS before blocking (3x 15 min) with 10% (v/v) FCS in PBS. Slides were incubated for 3 hr at room temperature with the primary antibody (anti-LAMP-1 monoclonal antibody; hybridoma supernatant) containing 0.05% (w/v) digitonin. Slides were then washed three times for 5 min and then incubated for one hour in the dark at 4°C with the FITC conjugated anti-mouse Ig (Silenus) which had been absorbed against ovalbumin/BSA coupled Affigel (2 mg/ml each). Slides were finally washed three times for 5 min with PBS, coverslips mounted in 50% (v/v)

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glycerol containing 2% (w/v) DABCO (diazabicyclo[2.2.2.]octane) and examined by epifluorescent microscopy.

Results (Lysosomal Biogenesis is Up-Regulated with Sucrosome Formation):

- 5 Sucrosomes in skin fibroblasts, were visible by light microscopy within 24 hr of introducing sucrose into the culture media. A series of sucrose concentrations (50 mM, 100 mM, 150 mM, 200 mM, and 250 mM sucrose) were compared for their effect on lysosomal enzyme expression and cell morphology. Skin fibroblasts were grown as contact inhibited, confluent, normal cell monolayers, which enabled them to be studied for many days without passaging. Fibroblasts
10 were incubated in the various sucrose concentrations for 7 days, after which they were harvested and lysosomal enzymes assayed. Cells incubated in 200 mM and 250 mM sucrose-containing media showed signs of osmotic stress after 7 days. A sucrose concentration of 100 mM was found to be optimal in that cells showed no signs of osmotic stress and lysosomal enzyme levels were found to be maximally elevated (results not shown), and 100 mM sucrose in the culture
15 media was routinely used for sucrosome induction.

- Time-course experiments were performed in which fibroblasts were incubated in the presence or absence of 100 mM sucrose for 1-28 days. Cells were harvested at 1, 2, 4, 7, 14, 21, and 28 days after being cultured in either sucrose-containing media or normal media.
20 Electron microscopy of cells grown in sucrose containing media, showed a dramatic increase in sucrosomes after only 24 hr, with a further increase in storage during the time-course (Fig. 6, Panels A,B,C,D). Concurrent immunofluorescent labeling of these cells with an anti-LAMP-1 monoclonal antibody, showed an increase in fluorescent labeling associated with lysosomes (Fig. 6, Panels E,F,G,H).

25

- The level of LAMP-1 protein increased 25-fold in the presence of sucrose over a 21 day period (Fig. 7). Immunofluorescent localisation of LAMP-1 in sucrosome containing cells showed that it was present in the sucrosome membrane with no significant elevation at the plasma membrane (Fig. 6, Panels E,F,G,H). The increase in LAMP-1 also correlated with the
30 formation of sucrosomes as determined by electron microscopy revealing not only an increase

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in size (compare Figs. 6A and 6B), but also an apparent increase in the number of these lysosomal storage vacuoles (compare Fig. 6B and 6D), with sucrosomes occupying most of the cytoplasm by days 21-28. However, although the presence of sucrosomes was evident after one day, both by electron microscopy (Fig. 6B) and immunofluorescent labeling (Fig. 6F), no
5 corresponding increase in LAMP-1 was observed at this time (Fig. 7), suggesting an initial increase in sucrosome volume due to swelling. LAMP-1 was not elevated until day 4, with the highest elevation observed at day 21 (Fig. 7), which corresponds with the apparent increase in sucrosome number during this time (Fig. 6D).

10 Enzyme activity levels of acid phosphatase, β -hexosaminidase, α -iduronidase, α -mannosidase, galactosyltransferase and α -ketoglutarate dehydrogenase during the induction of sucrosomes were investigated. The levels of acid phosphatase, β -hexosaminidase and α -mannosidase within the cells, showed similar profiles to that seen for LAMP-1 but were elevated 3, 4 and 5-fold respectively after 21 days (Table 3). α -Iduronidase,
15 galactosyltransferase (golgi marker) and α -ketoglutarate dehydrogenase (mitochondrial marker) activity levels did not appear to be elevated during lysosomal storage. Protein and enzyme activity levels were also determined in the media (Table 4), where we observed that LAMP-1, β -hexosaminidase, α -mannosidase and 4-sulphatase were secreted from the cells. Whereas LAMP-1, β -hexosaminidase and 4-sulphatase displayed a corresponding increase in the levels
20 secreted when the cells were grown in sucrose containing media, the α -mannosidase showed no such increase. Comparison of the amount of α -mannosidase secreted, with the level present in the cell, indicates that the 5-fold elevation of α -mannosidase activity observed in sucrosome containing cells was not due to an increase in α -mannosidase synthesis but rather an increase in the proportion of α -mannosidase trafficked to the lysosome.

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TABLE 3

Protein/Enzyme Levels in Skin Fibroblasts at two weeks post confluency

Cell line	LAMP-1	β -Hex ^b	ACP ^b	α -Mann ^b	α -Idu ^b	4-Sul ^b
normal (n=4)	1	1	1	1	1	1
Pompe	5	2-3	1	2	1	2
Salla	7	2-3	2	2	2	2
MPS-II	2	2-3	2-3	2	1	2
MPS-VI	1	1	1	1	1	1
Sucrosome ^a	25	4	3	5	1	2

^a level of proteins/enzymes. 3 weeks after cells were placed in sucrose containing media.

^b β -hexosaminidase (β -Hex), acid phosphatase (ACP), α -mannosidase (α -Mann), α -iduronidase (α -Idu) and 4-sulphatase (4-Sul) activities

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TABLE 4

Protein Levels in Sucrose Containing Cells and Media

		LAMP-1 [total fluorescence units (x10 ⁶)] Cell : Media ^c	β -Hex ^a (nmol/min) Cell : Media ^c	α -Mann ^a Cell : Media ^c	4-Sul ^a Cell : Media ^c
5	Day14 (S-) ^b	3 : 7	9 : 38	0.2 : 67	24 : 86
	Day 35 (S-) ^b	6 : 7	19 : 24	0.6 : 45	18 : 89
	Day 14 (S+) ^b	14 : 9	34 : 12	1.3 : 28	52 : 163
	Day 21 (S+) ^b	20 : 11	36 : 23	1.0 : 35	52 : 307
10	Day 28 (S+) ^b	51 : 22	91 : 113	2.4 : 34	63 : 651
	Day 35 (S+) ^b	58 : 31	114 : 153	3.1 : 37	125 : 1091
	Day 15 (INV) ^b	8 : 5	55 : 8	1.6 : 8	55 : 13
	Day 18 (INV) ^b	3 : 5	37 : 37	0.7 : 45	41 : 126
15	Day 21 (INV) ^b	2 : 7	45 : 49	0.6 : 57	49 : 240
	Day 28 (INV) ^b	1 : 6	57 : 43	0.5 : 67	24 : 163
	Day 35 (INV) ^b	2 : 7	42 : 24	0.4 : 51	61 : 179
	Day 15 (N) ^b	13 : 4	27 : 1	0.9 : 15	36 : 11
20	Day 18 (N) ^b	12 : 10	33 : 8	1.0 : 31	48 : 121
	Day 21 (N) ^b	9 : 13	48 : 22	0.9 : 31	67 : 265
	Day 28 (N) ^b	4 : 11	28 : 22	0.4 : 39	58 : 381
	Day 35 (N) ^b	4 : 8	30 : 21	0.3 : 68	70 : 414

25 ^a β -Hex; β -hexosaminidase; α -Mann. α -mannosidase; 4-Sul, 4-sulphatase.

^b S-, cells grown in BME media (no sucrose) throughout the time-course; S+, cells grown in BME media containing 100 mM sucrose; INV. cells corrected with 0.1 mg/ml invertase; N, cells corrected by being placed on BME media (no sucrose). Cells were fed by medium change every 7 days.

^c activities present in media represent total activity secreted since the last median change (i.e. 7 days in all cases except for day 15 and day 18 which were 1 day and 4 days post media change, respectively).

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EXAMPLE 9**Sucrosome Dissipation as a model of the Correction of a
Lysosomal Storage Disorder**

5 Cell culture methods, preparation of extracts, protein/enzyme assays, immunoquantification of Lamp-1, electron microscopy and immunofluorescence were performed as described in Example 8.

10 In order to study the effects of storage correction upon lysosomal biogenesis, fibroblasts were incubated in the presence or absence of 100 mM sucrose for 14 days, at that time cells were changed to either normal media: media containing invertase; or continued on sucrose-containing media. Cells that were grown in normal media from day 0 were continued in this media.

15 Electron microscopy verified that cells grown in sucrose-containing media for 14 days contained sucrosomes (Figs. 8A & 8B) and sucrosomes continued to accumulate to day 28 (Fig. 6D). The number of sucrosomes were markedly reduced within 24 hr (Figs. 8C & 8D) of the sucrosome-containing cells being returned to either normal media or to media containing invertase. The cells returned to normal within 4 days (day 18 of the time-course) (Figs. 8E & 20 8F). Sucrosome-containing cells placed on invertase, showed a faster correction with a greater decrease in the number of sucrosomes within 24 hr.

Immunofluorescence studies also showed a decrease in LAMP-1 fluorescent labelling after correction. This labelling was distinctively lysosomal (LAMP-1 positive, MPR negative), 25 with little or no cell surface staining detected. A decrease in LAMP-1 immunofluorescence was observed within 24 hr of cells being transferred to normal media or invertase, with the LAMP-1 labeling observed at this time point, similar to that seen in the cells which were grown continuously on normal media (data not shown).

30 The level of LAMP-1 protein increased 28-fold in cells grown in the presence of sucrose

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over a 35 day time-course (Fig. 9). This level returned to normal within 3 days, after sucrosome loaded cells were switched to media containing invertase. However, cells placed on normal media, required 7-14 days to normalise the level of LAMP-1.

5 Enzyme levels in cells that were grown in sucrose-containing media for 14 days and subsequently placed on normal media required 7 to 14 days to return to normal levels. As with LAMP-1, cells switched to invertase containing media showed the most dramatic change in enzymes levels, with levels decreasing to normal or below normal within 1-4 days. These invertase results correlated strongly with the formation and dissipation of storage vacuoles as
10 observed with electron microscopy and immunofluorescence, whereas the cells placed on normal media showed a lag time between dissipation of vacuoles and decrease in enzyme/protein levels.

 During correction of sucrosome containing cells, LAMP-1 and lysosomal enzyme
15 activities were determined in the cells and media (Table 4). This shows that when cells were corrected with invertase, total levels of LAMP-1 decreased from 14 units (in cells prior to correction, day 14 (+sucrose)) to 13, 8, and 9 units (total in cells plus media, at 15, 18 and 21 days, respectively), but increased over the same period when cells were switched to BME media (no sucrose). In contrast, the total enzyme activity levels increased, during correction with either
20 method.

EXAMPLE 10

Lysosomal Protein/Enzyme levels in LSD Affected Skin Fibroblasts

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Cell culture methods, preparation of extracts, protein/enzyme assays, immunoquantification of Lamp-1, electron microscopy and immunofluorescence were performed as described in Example 8.

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Normal skin fibroblast cell lines used in this study, included SF3921, SF4117, SF4153, SF4204 and SF4227. The affected cell lines cultured included SF3960 for Pompe disease, SF1594 for Salla disease, SF1779 for MPS-II and SF3168 for MPS-VI. Cell lines refer to the National Referral Laboratory cell bank held in the Department of Chemical Pathology, Women's and Children's Hospital, Adelaide, Australia.

An elevation in lysosomal enzymes, similar to the increase seen with sucrose induction, was also observed in LSD patient cell lines. In this study four LSD fibroblast cell lines (Pompe, Salla disease, MPS-II and MPS-VI) and four different normal control fibroblast cell lines were investigated.

Immunofluorescence studies showed a high level of LAMP-1 staining in Pompe cells (Fig. 10A) when compared to normal control fibroblasts (Fig. 6A). Salla, MPS-II and MPS-VI cells showed lower levels of LAMP-1 immunofluorescence, but still greater than the control cells. In all four disease cell lines, this labeling was associated with intracellular organelles (Fig. 10). Electron microscopy verified the presence of a large number of lysosomal storage vacuoles associated with Pompe, Salla and MPS-II cells but relatively few associated with MPS-VI cells (Fig. 11). Immunofluorescence studies with anti-LAMP-1 antibody revealed an increase in staining with time, once the cells reached confluency. Electron microscopy further verified elevated levels of lysosomes in Salla, Pompe and MPS-II cells, when compared to normal fibroblasts, over a 14 day post confluent period (results not shown). MPS-VI cells showed no apparent increase in storage vacuoles over the 14 day incubation.

The Salla disease cells displayed the highest elevation of LAMP-1 protein (7-fold above normal controls) which remained stable over the 14 day time-course. Pompe cells also produced a higher level of LAMP-1 (5-fold above normal) when compared to the level of LAMP-1 in normal fibroblast. A 2-fold increase in LAMP-1 was observed in the MPS-II disease cells throughout the 14 day time-course but no significant elevation was seen in the MPS-VI cells.

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Lysosomal enzymes were also elevated in LSD affected cell lines. β -Hexosaminidase was elevated 2-3 fold in the Salla, Pompe, and MPS-II cell lines, when compared to enzyme levels in normal skin fibroblasts (Table 3). α -Mannosidase was also up-regulated up-to 2-fold in Salla, Pompe and MPS-II cells. Acid phosphatase was observed to be increased up-to 2-fold in Salla and MPS-II cells. however no elevation of acid phosphatase was measured in Pompe cells. α -Iduronidase was only found to be elevated (2-fold) in the Salla disease cells, with no increase determined in Pompe, MPS-II, and MPS-VI. Of the four disease cell lines studied, MPS-VI showed no elevation in the levels of the four soluble lysosomal enzymes assayed, when compared to enzyme levels in normal skin fibroblasts (Table 3). No difference in the level of mRNA encoding 4-sulphatase was observed in any of the affected cell lines when compared to the level produced in normal fibroblast cells. However, these affected cell lines were only studied over a 14 day time course, whereas the increase in 4-sulphatase mRNA detected in the sucrosome time-course was not observed until later, at days 21-28. A slight increase (less than 2-fold) was observed in 4-sulphatase activity levels in Salla, Pompe and MPS-II cell lines, similar to the sucrosome time-course (Table 3).

EXAMPLE 11

Lysosomal Protein/Enzyme levels in LSD Affected Skin Fibroblasts after Correction

Cell culture methods, preparation of extracts, protein/enzyme assays, immunoquantification of Lamp-1, electron microscopy and immunofluorescence were performed as described in Example 8.

Correction of the storage in patient cell lines was achieved by the addition of the recombinant enzymes into the culture media of cells. All recombinant enzyme concentrations used have been previously shown to result in the correction of lysosomal storage (Fuller *et al*, 1995; Bielicki *et al*, 1993; Anson *et al*, 1992). Electron microscopy confirmed correction in Pompe and MPS-II affected cells, with the size of storage vacuoles dramatically decreasing within 7 days after the addition of the enzyme. In the case of MPS-VI affected cells, storage was observed to be minimal, with only a few storage vacuoles present, as detected by electron

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microscopy. Hence no change was observed upon correction. Immunofluorescence studies with anti-LAMP-1 monoclonal antibody also demonstrated a decrease in LAMP-1 staining as a consequence of correction. Pompe affected cells showed intense fluorescent labeling with the LAMP-1 antibody (Fig. 10A), however within 3 days of correction. Pompe cells were
5 normalised, resulting in a decrease in fluorescent labeling (not shown). Correction and subsequent normalisation of MPS-II and MPS-VI cells also showed decrease in lysosomal labeling within 7 days.

As a result of Pompe and MPS-II correction, the elevated LAMP-1 protein levels were
10 observed to decrease to the level of normal controls within 3-7 days (Fig. 12). The activity levels of the lysosomal enzymes acid phosphatase, β -hexosaminidase, α -iduronidase and α -mannosidase, were all elevated 2-3 fold in the MPS-II and Pompe disease cells, when compared to normal fibroblast levels. Correction of MPS-II with iduronate-2-sulphatase, resulted in these lysosomal enzymes returning to the normal range within 7 days of correction. Surprisingly no
15 significant change in the elevated lysosomal activity levels was observed with the correction of the Pompe disease cells, although a decrease in storage vacuoles was detected with electron microscopy and immunofluorescence studies, as well as an accompanying decrease in the LAMP-1 levels. MPS-VI disease cells did not display elevated levels in any of the lysosomal enzymes assayed and no increase in LAMP-1 normally observed with other storage disorders
20 (results not shown). Thus, only a small difference existed between affected and corrected cells and no significant change was detected as a result of correction.

REFERENCES

1. Anson, D.S., Taylor, J.A., Bielicki, J., Harper, G.S., Peters, C., Gibson, G.J., and Hopwood, J.J. (1992) Correction of human mucopolysaccharidosis type VI fibroblasts with recombinant N-acetylgalactosamine-4-sulphatase. *Biochem. J.* 284, 789-794.
2. Avila, J.L. and Convit, J. (1973) Characterisation and properties of α -D-mannosidase of human polymorphonuclear leucocytes. *Clin. Chim. Acta* 51, 335-343.
3. Bielicki, J., Hopwood, J.J., Wilson, P.J., and Anson, D.S. (1993) Recombinant human iduronate-2-sulphatase: correction of mucopolysaccharidosis-type II fibroblasts and characterization of the purified enzyme. *Biochem. J.* 289, 241-246.
4. Brooks, D.A., Gibson, G.J., and Hopwood, J.J. (1994) Immunochemical characterization of feline and human N-acetylgalactosamine 4-sulfatase. *Biochem. Med. Met. Biol.* 53, 58-66.
5. Bullock *et al.* (1982, 1983, 1984) *Techniques in Immunocytochemistry* Academic Press, Orlando.
6. Chard. (1986) *In: An Introduction to radio immunoassay and related techniques.* Elsevier Science, Amsterdam, The Netherlands.
7. Clements, P.R., Brooks, D.A., Saccone, G.T.P., and Hopwood J.J. (1985) Human α -L-iduronidase. 1. Purification, monoclonal antibody production, native and subunit molecular mass. *Eur. J. Biochem.* 152, 21-28.
8. Cole *et al.* (1985) *In: Monoclonal antibodies in cancer therapy*, Alan R. Bliss Inc. pp 77-96.
9. Crawley A.C. *et al.* Enzyme replacement therapy in a feline model of Maroteaux-Lamy

syndrome. J. Clin. Invest. 1996; 97:1864-1873.

10. Crawley A.C. Neidzielski K.H., Issac E.L., Davey R.C.A., Byers, S., Hopwood, J.J.
Enzyme replacement therapy from birth in a feline model of mucopolysaccharidosis type VI.J.

5 Clin. Invest. 1997; 99: 651-662.

11. Dahlgren, C., Carlsson, S.R., Karlsson, A., Lundqvist, H., and Sjölin, C. (1995) The
lysosomal membrane glycoproteins LAMP- 1 and LAMP-2 are present in mobilizable
organelles, but are absent from the azurophilic granules of human neutrophils. Biochem. J. 311,
10 667-674.

12. Diamandis, E.P. Immunoassays with time-resolved fluorescence spectroscopy:
principles and applications. Clin. Biochem. 1988; 21:139-150.

15 13. Fuller, M., van der Ploeg, A., Reuser, A.J.J., Anson, D.S., and Hopwood, J.J. (1995)
Isolation and characterisation of a recombinant, precursor form of lysosomal acid α -glucosidase.
Euro. J. Biochem. 234, 903-909.

14. Gahl, W.A., *et al.* Cysteamine therapy for children with nephropathic cystinosis. N.
20 Engl. J. Med. 1987; 316:971-977.

15. Haskins, M.E., Otis, E.J., Hayden, J.E., Jezyk, P.F., Stramm, L. Hepatic storage of
glycosaminoglycans in feline and canine models of mucopolysaccharidoses I, VI and VII. Vet.
Pathol. 1992; 29:112-119.

25

16. Hemmila, I., Lanthanides as probes for time-resolved fluorometric immunoassays.
Scand. J. Clin. Lab. Invest. 1988; 48:389-399.

17. Hoogerbrugge, P.M., *et al.* Allogeneic bone marrow transplantation for lysosomal
30 storage diseases. The European Group for Bone Marrow Transplantation [see comments].

Lancet 1995; 245:1398-1402.

18. Hopwood, Muller, V., Harrison, J.R., Carey, W.F., Elliott, H., Robertson, E.F. and Pollard, A.C. (1982) Enzymatic diagnosis of the mucopolysaccharidoses. Med. J. Aust. 1982; 5 1:257-260.

19. Hopwood, J.J., et al. Long-term clinical progress in bone marrow transplanted mucopolysaccharidosis type I patients with a defined genotype. J. Inherit. Metab. Dis. 1993; 16:1024-1033.

10

20. Huse *et al.* (1989) Science 246: 1275-1281.

21. Jones, M.Z., Kennedy, F.F., Caprine beta-mannosidosis: aberrant phenotype in a 5-month-old euthyroid animal. J. Inherit. Metab. Dis. 1993; 16:910-911.

15

22. Kampine, J.P., Brady, R.O., Kanfer, J.N., Feld M., Shapir, D. Diagnosis of Gaucher disease and Niemann-Pick disease with small samples of venous blood. Science 1967; 155:86-88.

20 23. Kohler and Milstein (1975) Nature, 256: 495-499.

24. Kozbor *et al.* (1983) Immunol. Today 4: 72;

25. Kolodny, E.H., and Mumford, R.A. (1976) Human leukocyte acid hydrolases: Characterisation of eleven lysosomal enzymes and study of reaction conditions for their automated analysis. Clin. Chim. Acta. 70, 247-257.

26. Leaback, D.H. and Walker, P.G. (1961) Studies on glucosaminidase. IV. The fluorometric assay of N-acetyl- β -glucosaminidase. Biochem. J. 78, 151-156.

30

- 45 -

27. Markello, T.C., Bernardini, I.M., Gahl, W.A. Improved renal function in children with cystinosis treated with cysteamine. *N. Engl. J. Med.* 1993; 328:1157-1162.
28. Meikle, P.J., Whittle, A.M., Hopwood, J.J. Human acetyl-coenzyme A: α -glucosaminide N-acetyltransferase. Kinetic characterisation and mechanistic interpretation. *Biochem. J.* 1995; 308:327-333.
29. Neufeld, E.F., Meunzer, J. The Mucopolysaccharidosis. In Scriver, C.R., Beaudet, A.C., Sly, W.S., Valle, D., eds. *The Metabolic and Molecular Basis of Inherited Disease* (Seventh Edition). New York: McGraw-Hill Inc. 1995: Vol II, 2465-2494.
30. Otterbach, B., Stoffel, W. Acid sphingomyelinase-deficient mice mimic the neurovisceral form of human lysosomal storage disease (Niemann-Pick disease). *Cell* 1995; 81:1053-1061.
31. Rome, L.H., Garvin, A.J., Allietta, M.M. and Neufeld, E.F. (1979). Two species of lysosomal organelles in cultured human fibroblasts. *Cell* 17, 143-153.
32. Sands, M.S. et al. Enzyme replacement therapy for murine mucopolysaccharidosis type VII. *J. Clin. Invest.* 1994; 93:2324-2331.
33. Singh, H, Derwas, N. and Poulos, A. (1987). Very long chain fatty acid beta-oxidation by subcellular fractions of normal and Zellweger syndrome skin fibroblasts. *Arch Biochem Biophys* 257, 302-314.
34. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76-85.
35. Taylor, R.M., Stewart, G.J., Farrow, B.R. Improvement in the neurologic signs and

- 46 -

storage lesions of fucosidosis in dogs given marrow transplants at an early age. Transplant. Proc. 1989; 21:3818-3819.

36. Thompson, J.N., Jones, M.Z., Dawson, G., Huffman, P.S. N-acetylglucosamine 6-
5 sulphatase deficiency in a Nubian goat: a model of Sanfilippo syndrome type D
(mucopolysaccharidosis IIID)l. J. Inherit, Metab. Dis. 1992; 15:760-768.
37. Tijssen (1985) Practice and Theory of enzyme immunoassays: Laboratory Techniques
in Biochemistry and Molecular Biology, Elsevier Science, Amsterdam, The Netherlands.
- 10 38. Zhou, X.Y. et al. Mouse model for the lysosomal disorder galactosialidosis and
correction of the phenotype with overexpressing erythroid precursor cells. Genes Dev. 1995;
9:2623-2634.
- 15 39. . Zola, H. and Brooks, D. Techniques for the Production and characterization of
Monoclonal Hybridoma Antibodies. In Hurrell J.G.R., ed. Monoclonal Hybridoma
Antibodies: Techniques and Application. Boca Raton: CRC Press, Inc. 1982: 1-57.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of detecting a lysosomal storage disorder (LSD), monitoring the progress of an LSD or the efficacy of treatment of an LSD in a human or animal subject, said method comprising assaying the level of an LSD marker in a biological sample derived from said patient, wherein said LSD marker is an enzyme, a polypeptide or a protein which is associated with the occurrence, development or onset of said LSD, or an immunologically interactive homologue, analogue or derivative thereof.
2. The method according to claim 1 wherein the step of assaying the level of an LSD marker comprises measuring the enzyme activity of said LSD marker in the biological sample.
3. The method according to claim 1 wherein the step of assaying the level of an LSD marker comprises contacting the biological sample with one or more immunointeractive molecules specific for said LSD marker for a time and under conditions sufficient for the formulation of a complex to occur.
4. The method according to claim 3 wherein the immunointeractive molecule is an antibody molecule which binds to the LSD marker.
5. The method according to claim 4 wherein the antibody molecule is a monoclonal antibody which binds to the LSD marker.
6. The method according to any one of claims 3 to 5 wherein the immunointeractive molecule or antibody molecule is labeled with a reporter molecule.

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7. The method according to any one of claims 3 to 5 further comprising the step of contacting the complex formed between the LSD marker and the immunointeractive molecule or antibody molecule with a labeled immunointeractive molecule for a time and under conditions sufficient for binding to occur.
8. The method according to claim 7 wherein the labeled immunointeractive molecule is labeled with a reporter molecule.
9. The method according to claim 6 or 8 wherein the reporter molecule is an enzyme, fluorophore or radionuclide molecule.
10. The method according to claim 9 wherein the enzyme, fluorophore or radionuclide molecule is selected from the list comprising horseradish peroxidase, glucose oxidase, β -galactosidase, alkaline phosphatase, fluorescein, Eu^{3+} or other lanthanide metal, or rhodamine.
11. The method according to claim 10 wherein the fluorophore molecule is Eu^{3+} .
12. The method according to any one of claims 1 to 11 wherein the enzyme, polypeptide or protein is a lysosomal enzyme, polypeptide or protein or an enzyme, polypeptide or protein which at least is transported to the lysosome or accumulates in the lysosome.
13. The method according to any one of claims 1 to 11 wherein the enzyme, polypeptide or protein is selected from the list comprising Lamp-1, Lamp-2, Limp-II, mannose-6-phosphate receptor, α -L-iduronidase, 4-sulphatase, acid phosphatase (ACP), β -hexosaminidase, and α -mannosidase.
14. The method according to claim 13 wherein the enzyme, polypeptide or protein is Lamp-1.

15. The method according to claim 13 wherein the enzyme, polypeptide or protein is Lamp-2.
16. The method according to any one of claims 1 to 15 wherein the LSD is selected from the list set forth in Table 1.
17. The method according to claim 16 wherein the LSD is selected from the list comprising MPS I, MPS II, Gaucher disease, Pompe disease and Salla's disease.
18. The method according to claim 17 wherein the LSD marker is Lamp-1.
19. The method according to any one of claims 1 to 18 wherein the biological sample is blood, plasma, fibroblast cell or fibroblast cell culture or cell extract thereof.
20. The method according to claim 19 wherein the fibroblast cell or fibroblast cell culture is a skin fibroblast or skin fibroblast cell culture or a cell extract thereof.
21. The method according to claim 20 wherein the fibroblast cell or fibroblast cell culture is a Pompe, Salla, MPS II or MPS VI fibroblast cell or cell culture or a cell extract thereof.
22. An antibody molecule which binds to an LSD marker or a hybridoma or other cell line producing same when used in the method according to any one of claims 1 to 21.
23. The antibody molecule according to claim 22 wherein the LSD marker is Lamp-1 or Lamp-2.

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24. The antibody molecule according to claim 22 further defined as a polyclonal antibody.

25. The antibody molecule according to claim 22 further defined as a monoclonal antibody.

26. The antibody molecule according to any one of claims 23 to 25 labeled with a reporter molecule.

27. The antibody molecule according to claim 26 wherein the reporter molecule is an enzyme, fluorophore or radionuclide molecule.

28. The antibody molecule according to claim 27 wherein the enzyme, fluorophore or radionuclide molecule is selected from the list comprising horseradish peroxidase, glucose oxidase, β -galactosidase, alkaline phosphatase, fluorescein, Eu^{3+} or other lanthanide metal, or rhodamine.

29. The antibody molecule according to claim 28 wherein the fluorophore molecule is Eu^{3+} .

30. A kit when used to detect a lysosomal storage disorder (LSD), or monitor the progress of an LSD, or monitor the efficacy of treatment of an LSD, said kit comprising an antigen which comprises an LSD marker enzyme, protein or polypeptide, or an immunologically interactive derivative, homologue or analogue thereof, and a first antibody molecule which is capable of binding to said antigen.

31. The kit according to claim 30 wherein the first antibody is a monoclonal antibody.

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32. The kit according to claim 30 wherein the first antibody is a polyclonal antibody.
33. The kit according to any one of claims 30 to 32 wherein the first antibody is labeled with a reporter molecule.
34. The kit according to claim 33 wherein the reporter molecule is an enzyme, fluorophore or radionuclide molecule.
35. The kit according to claim 34 wherein the enzyme, fluorophore or radionuclide molecule is selected from the list comprising horseradish peroxidase, glucose oxidase, β -galactosidase, alkaline phosphatase, fluorescein, Eu^{3+} or other lanthanide metal, or rhodamine.
36. The kit according to claim 35 wherein the fluorophore molecule is Eu^{3+} .
37. The kit according to any one of claims 30 to 36 further comprising a second antibody molecule which recognises the first antibody, wherein said second antibody is conjugated to a reporter molecule.
38. The kit according to claim 37 wherein the reporter molecule is an enzyme, fluorophore or radionuclide molecule.
39. The kit according to claim 38 wherein the enzyme, fluorophore or radionuclide molecule is selected from the list comprising horseradish peroxidase, glucose oxidase, β -galactosidase, alkaline phosphatase, fluorescein, Eu^{3+} or other lanthanide metal or rhodamine.
40. The kit according to claim 39 wherein the fluorophore molecule is Eu^{3+} .

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41. The kit according to claim 38 wherein, if the reporter molecule is an enzyme, said kit further comprises a substrate molecule specific for said enzyme.

42. The kit according to any one of claims 30 to 41 wherein the antigen is a lysosomal enzyme, polypeptide or protein or an immunologically interactive homologue, analogue or derivative thereof.

43. The kit according to claim 42 wherein the enzyme, polypeptide or protein is selected from the list comprising Lamp-1, Lamp-2, Limp-II, mannose-6-phosphate receptor, α -L-iduronidase, 4-sulphatase, acid phosphatase (ACP), β -hexosaminidase, and α -mannosidase.

44. The kit according to claim 43 wherein the antigen is Lamp-1 or an immunologically interactive derivative, homologue, or analogue thereof.

45. The kit according to claim 43 wherein the antigen is Lamp-2 or an immunologically interactive derivative, homologue, or analogue thereof.

46. The kit according to any one of claims 30 to 45 wherein the LSD is selected from the list set forth in Table 1.

47. The kit according to claim 46 wherein the LSD is selected from the list comprising MPS I, MPS II, Gaucher disease, Pompe disease and Salla's disease.

48. An isolated LSD marker enzyme, protein or polypeptide or an immunologically interactive homologue, analogue or derivative thereof when used in the method according to any one of claims 1 to 21.

49. The isolated LSD marker according to claim 48 selected from the list comprising Lamp-1, Lamp-2, Limp-II, mannose-6-phosphate receptor,

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α -L-iduronidase, 4-sulphatase, acid phosphatase (ACP), β -hexosaminidase and α -mannosidase.

50. The isolated LSD marker according to claim 49 further defined as Lamp-1.
51. The isolated LSD marker according to claim 49 further defined as Lamp-2.

[illegible]

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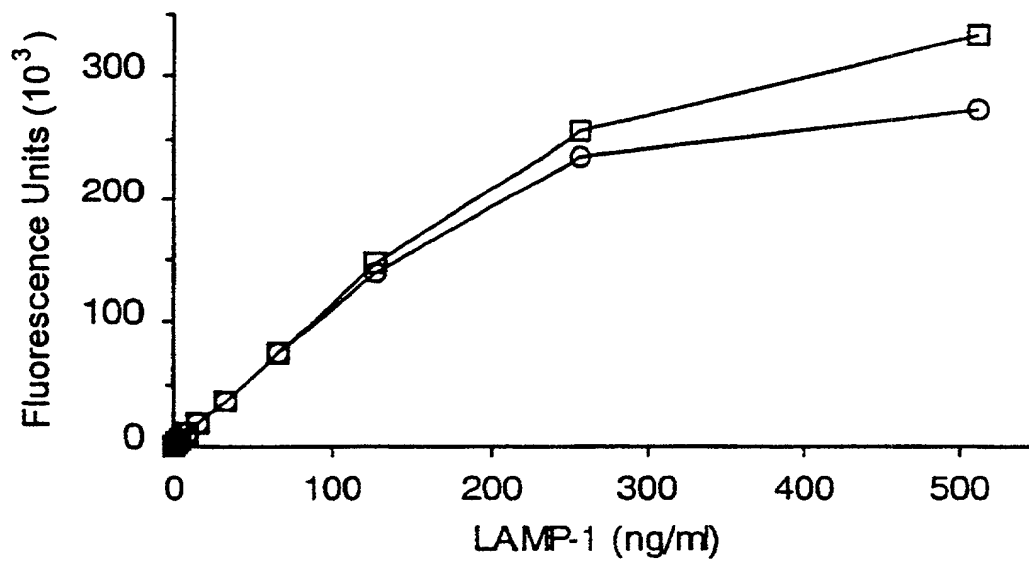


Figure 1

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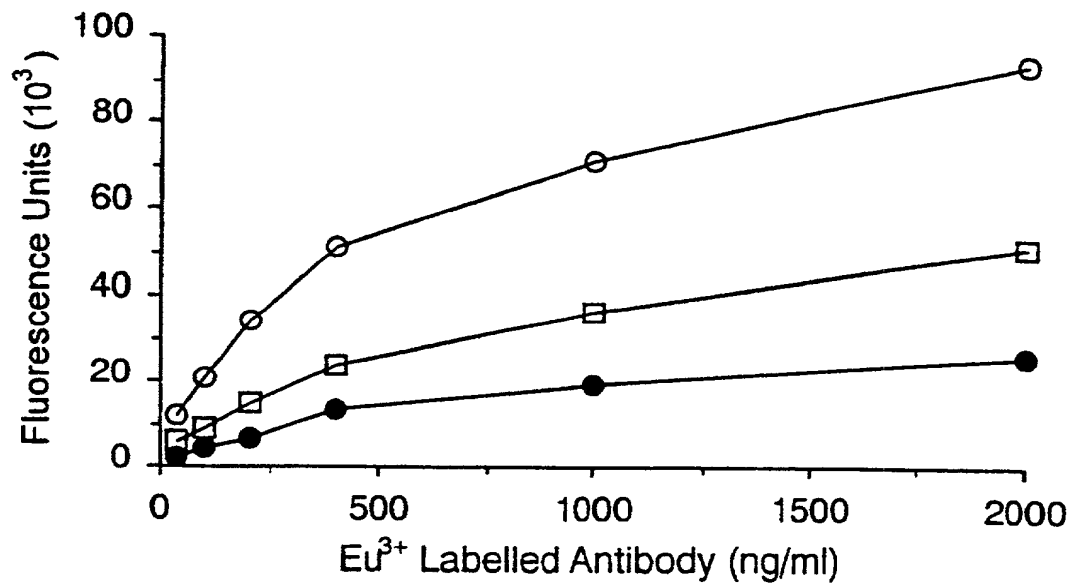


Figure 2

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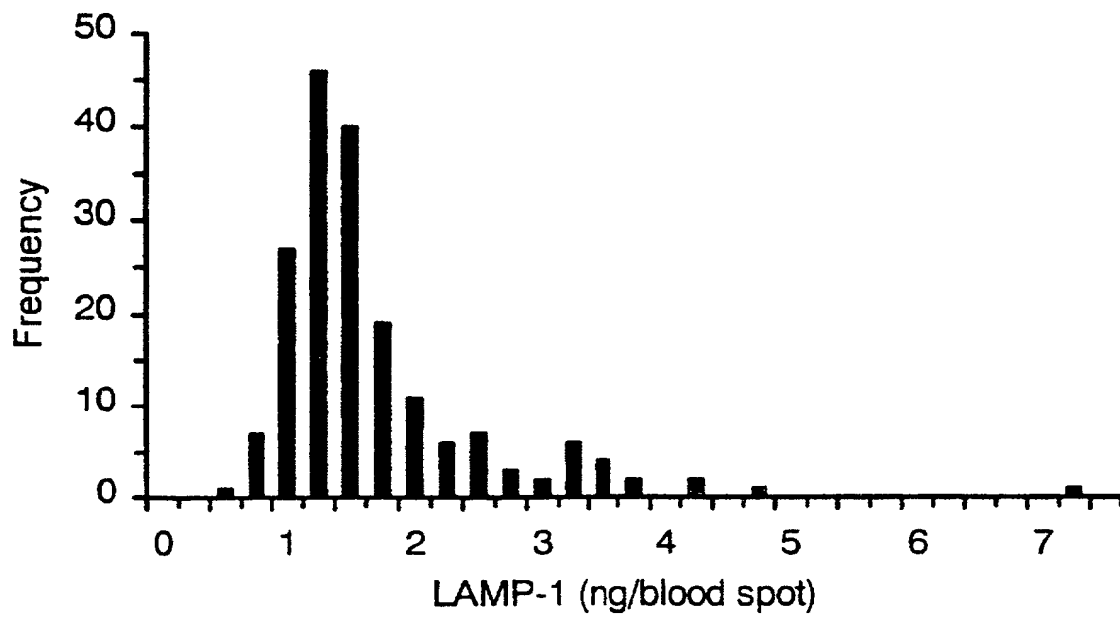


Figure 3

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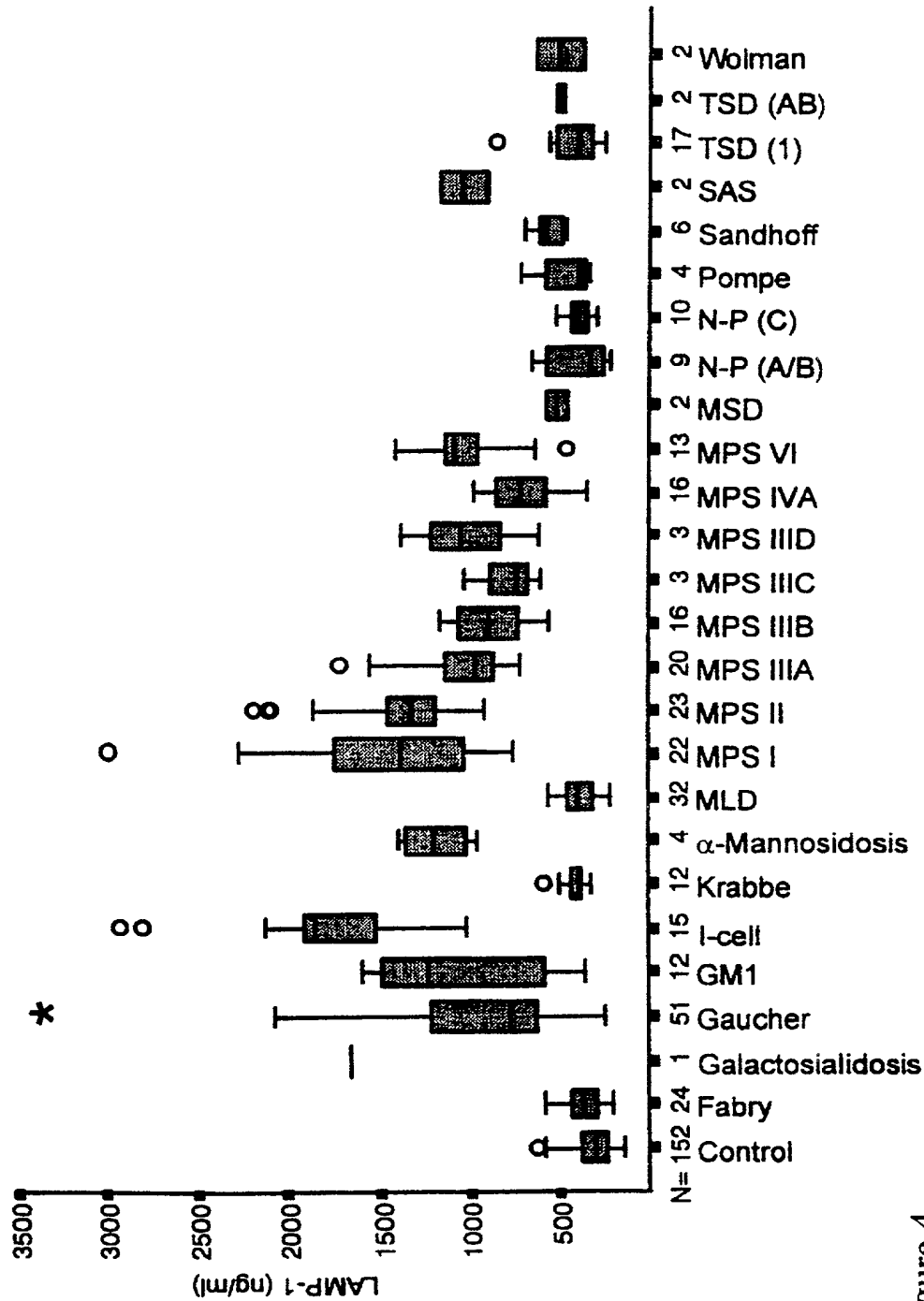


Figure 4

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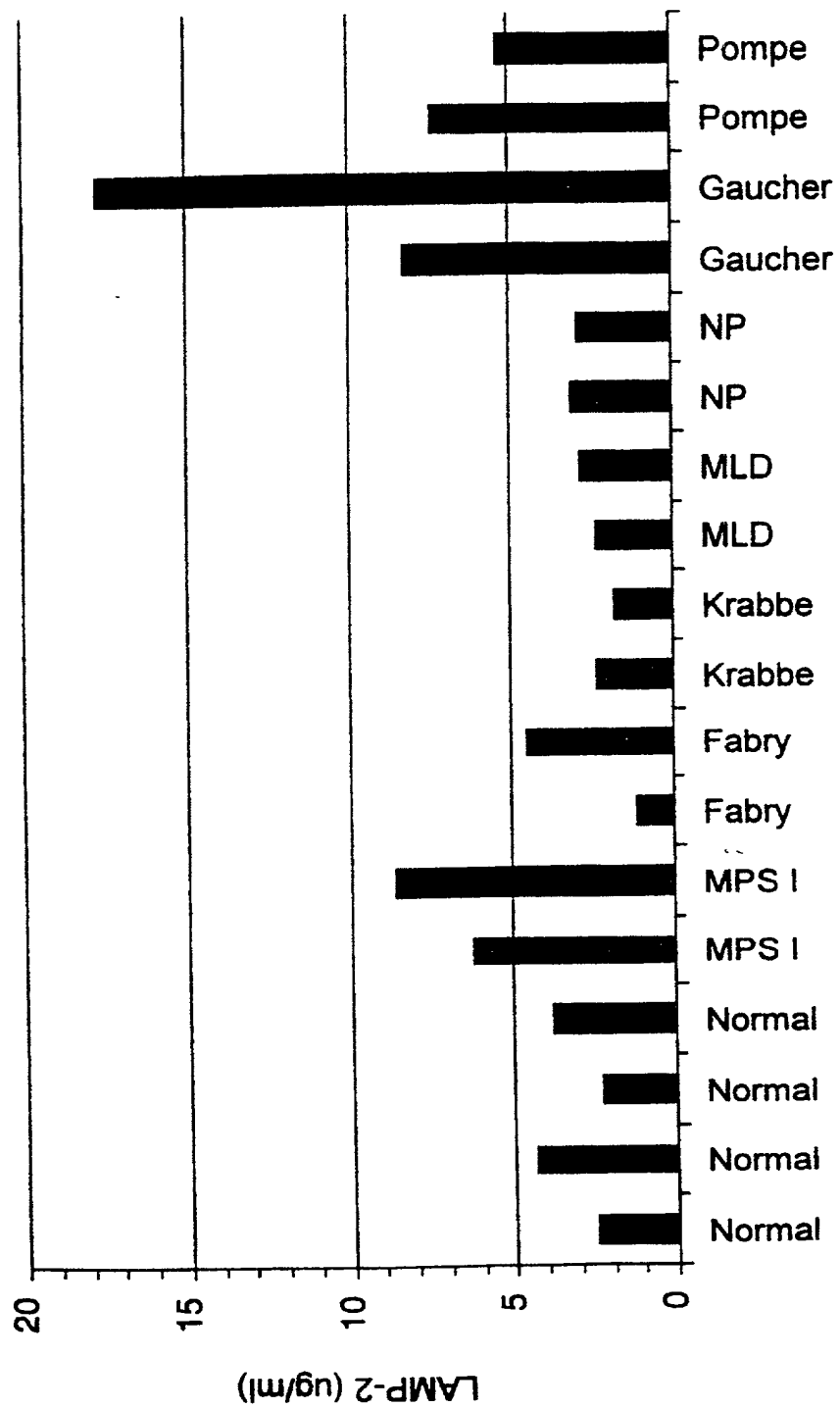


Figure 5

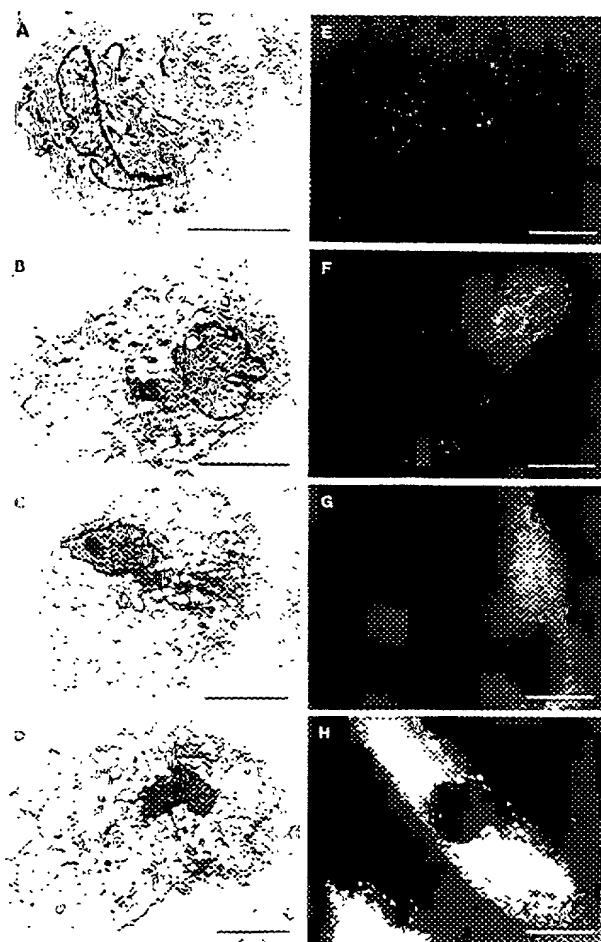


Figure 6

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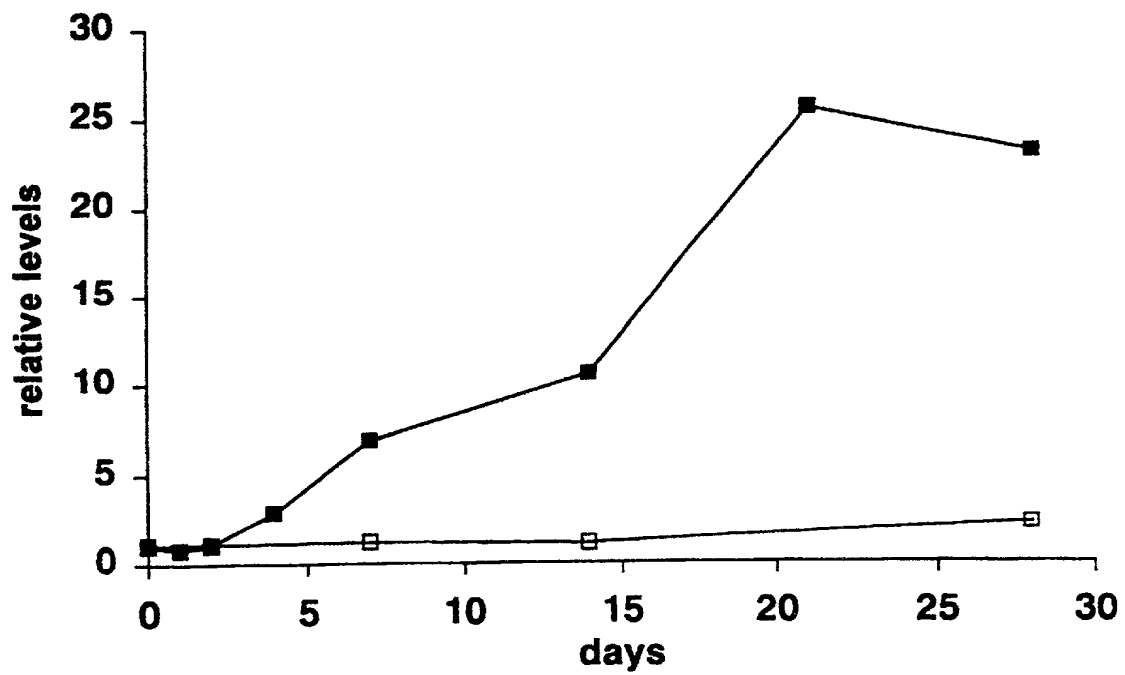


Figure 7

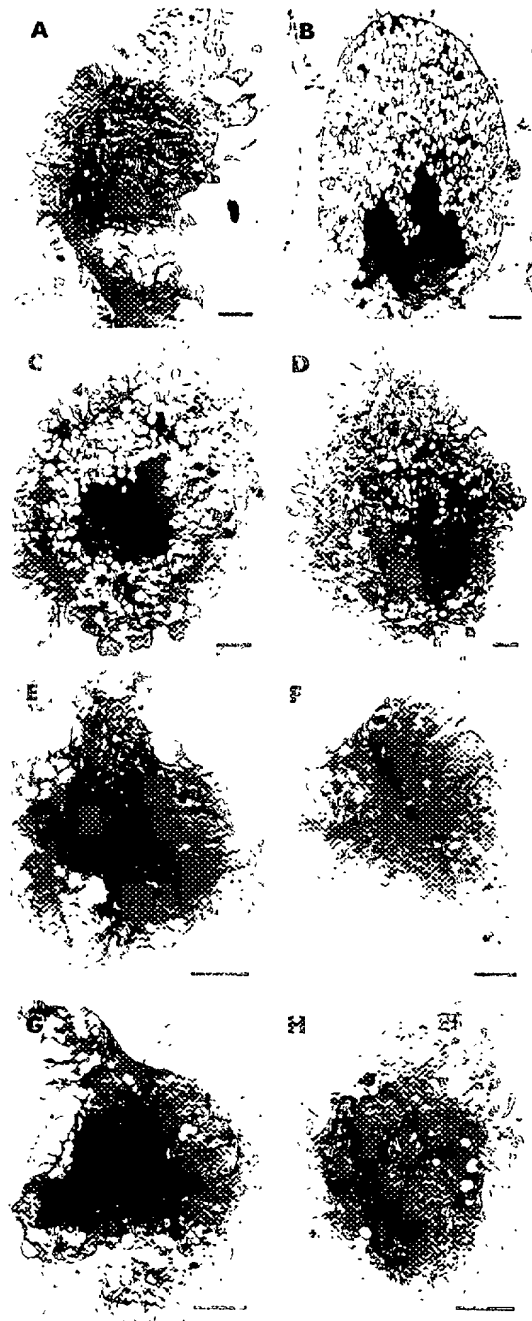


Figure 8

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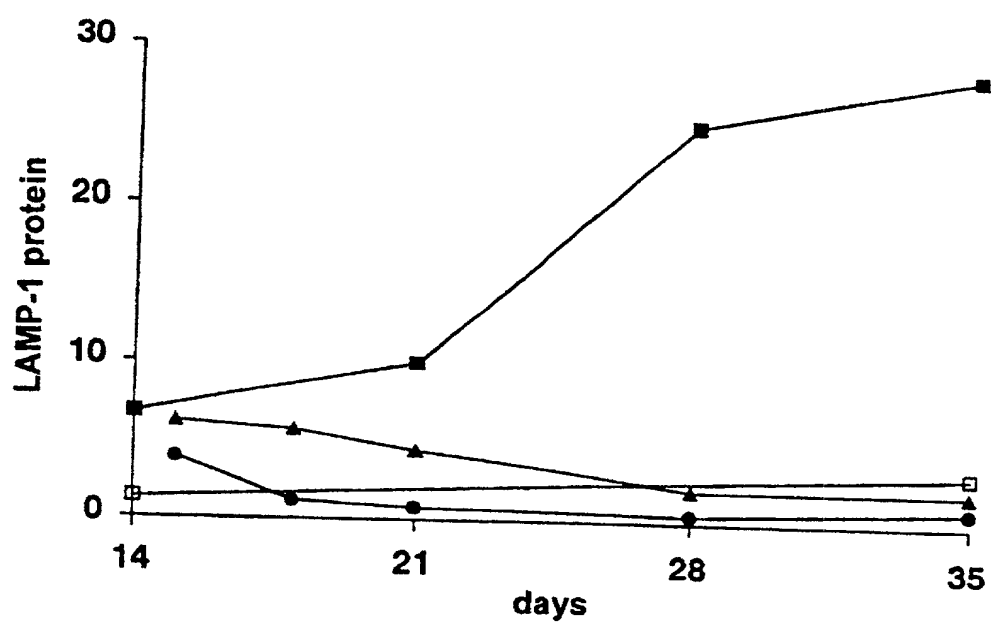


Figure 9

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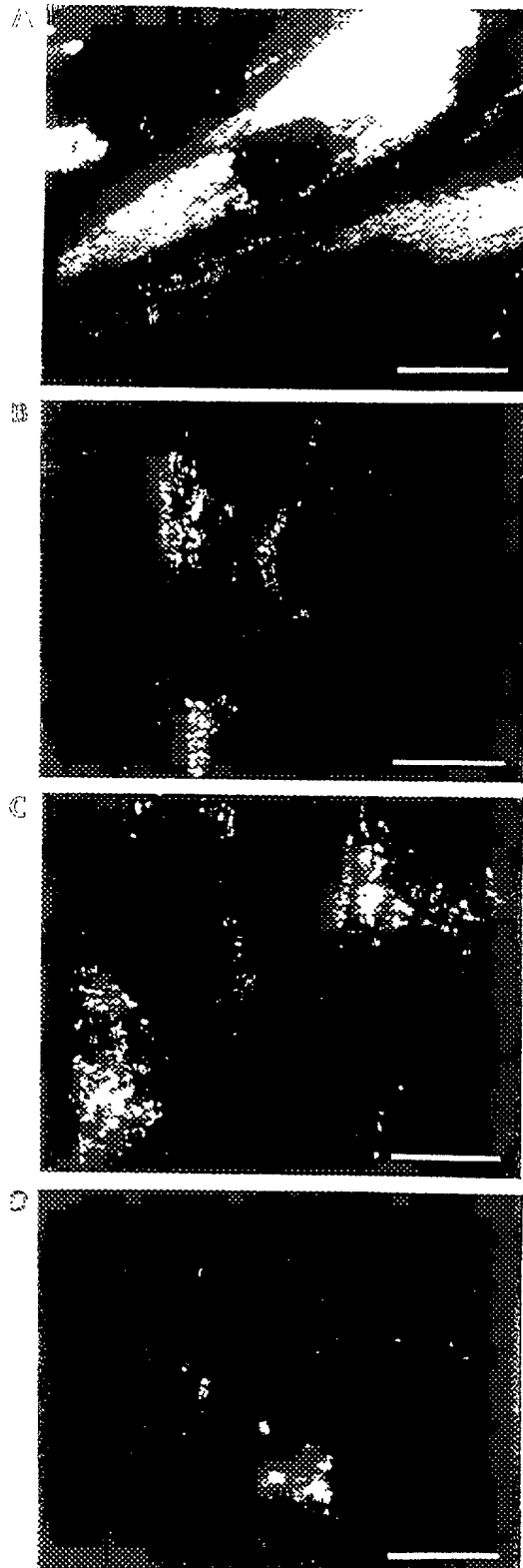


Figure 10

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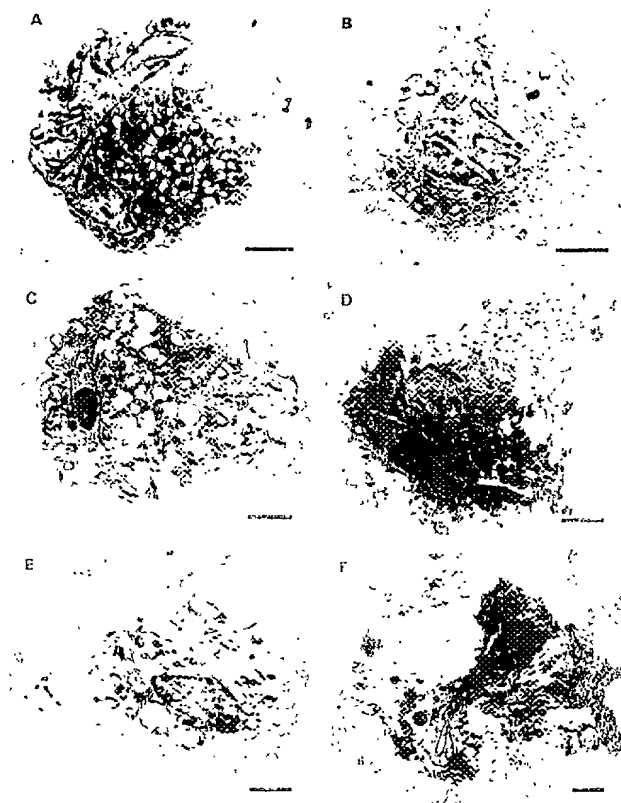


Figure 11

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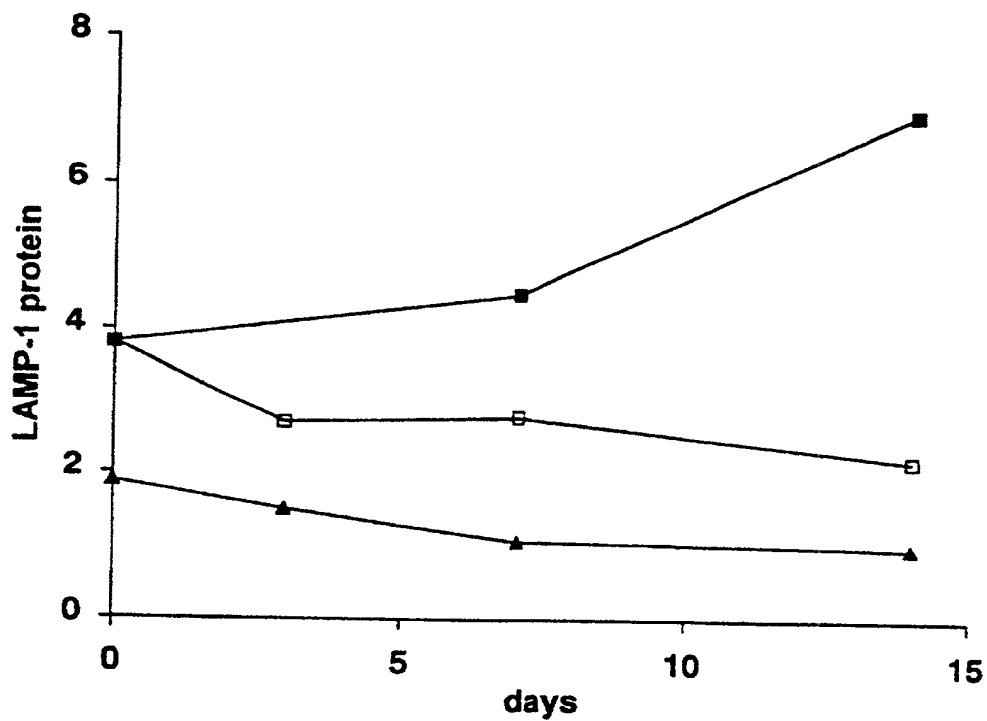


Figure 12

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below under my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
EARLY DETECTION OF LYSOSOMAL STORAGE DISORDERS

the Specification of which

☒ is attached hereto
☐ was filed on May 16, 1997
as Application Serial No. PCT/AU97/00304
and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified Specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

<u>APPLICATION</u> <u>NUMBER</u>	<u>PRIOR FOREIGN FILED APPLICATION(S)</u> <u>COUNTRY</u> <u>(MONTH/DAY/YYYY)</u>	<u>PRIORITY</u> <u>CLAIMED</u>
PN 9917	AUSTRALIA 17th May 1996	YES

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER(S)

FILING DATE (MM/DD/YYYY)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent <u>Application No.</u>	PCT Parent <u>Number</u>	Parent Filing <u>(MM/DD/YYYY)</u>	Parent Patent <u>Number (if applicable)</u>
	PCT/AU97/00304	16th May 1997	PN 9917

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so

made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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